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THE

BIOLOGICAL BULLETIN

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LONGITUDINAL AND TRANSVERSE DIVISION IN TWO CLOSELY RELATED FLAGELLATES

L. R. CLEVELAND

(From the Biological Laboratories, Harvard University, Cambridge, Massachusetts)

The two flagellates considered in this paper are closely related morphologically, and are probably of common origin; yet one divides longitudinally in a typical flagellate manner, and the other transversely, as in ciliates. In the latter, the anterior daughter cell retains the parent extranuclear organelles, while the other daughter develops new organelles at the posterior end. This organism may represent the beginning stage in the development of a new group of flagellates.

The chromosomes of both organisms are interesting because they are large, two in number (except in rare instances), clearly differentiated in size, persist through the interphase, show structural details plainly, divide in the telophase, and are unmistakably moved to the poles by the extranuclear chromosomal fibres.

The achromatic figure is also interesting because the fibres composing it are as distinct as the flagella which the organisms possess, and it is plain that they have the same individuality as the flagella. They arise from the centrioles, which follow the flagellar bands, and are sometimes so intermingled with the flagella that they can be distinguished from them only by their function in nuclear division.

Eleven species of *Spirotrichonympha* have been described from termites but, with the exception of *S. polygyra*, the species described by Cupp (1930), the descriptions are so brief that it is impossible to determine how many species are valid. And the description of Cupp left unanswered many vital questions concerning the structure and behavior of extranuclear organelles and the processes of nuclear division. Further, an examination of the protozoa of *Kalotermes* (*Paraneotermes* Light) *simplicicornis* (Banks), the termite harboring the *S. polygyra* described by Cupp, shows that her description dealt with two distinct organisms, and is therefore not only inadequate but, in many respects, inaccurate. However, it is not surprising that Cupp overlooked the fact that she was dealing with two organisms, because they are so much alike in some respects—and yet so different in others. If the number of flagellar bands is not considered, their differences may be seen only when the processes of cell division are studied. These processes Cupp states she did not understand because of failure to find a sufficient number of successive stages.

An examination of the interphase flagellates of this termite shows that the number of organisms with two spiral flagellar bands is about equal to the number with four spiral flagellar bands. This fact Cupp noted, but she supposed that those with two bands were recently divided four-banded forms which would soon develop two new bands. But the number of two-banded forms is just as great in preparations without dividing organisms as in those with them. This alone indicates that the two-banded forms are not young or developing four-banded forms.

There are also forms with three, five, and six spiral flagellar bands, but these are very rare in comparison with the two- and four-banded forms. In all hypermastigote flagellates occasional irregularities occur in the flagellated areas, and therefore one should expect to find a few organisms, when countless thousands are studied, whose spiral flagellar bands likewise deviate from the normal numbers. These abnormalities are probably due, at least in part, as unpublished observations on *Barbulanympha* show, to exceptional behavior of the centriole, the organelle from which the flagellated areas arise; sometimes, one centriole fails to produce another from itself during cell division, in which case one daughter cell gets only one centriole while the other gets two; and at other times, one centriole produces two new centrioles instead of one from itself, thus giving one daughter three centrioles and the other two.

Organisms with one flagellar band also occur, and in preparations containing many dividing individuals they are present in greater numbers than the forms with three, five, and six flagellar bands; some of these are probably the result of irregularities in the behavior of the centriole, but most of them, as will be made clear presently, are posterior daughters produced in the transverse division of the body of the forms with two spiral flagellar bands.

Even though Cupp considered the forms with two spiral flagellar bands to be identical with those having four bands, her species, *S. polygyra*, is valid for the forms with four bands because she considered four to be the normal number of bands. It is only necessary to give a name to the form with two bands. This, however, is not an easy task, for its relationship to previously described species and genera must be considered from three angles: should it be placed in any of the three genera which possess two flagellar bands; should *Spirotrichonympha*, which possesses four flagellar bands, be amended so as to receive it; or should a new genus be erected for it. Except for the fact that it has two instead of four flagellar bands, it is indistinguishable from *S. polygyra* when only interphase extranuclear organelles are considered, and its interphase nucleus does not differ greatly from that of *S. polygyra;* the four chromosomal coils are in two sheaths, while in *S. polygyra* each of the four chromosomal coils is in a separate sheath. This difference results from the failure of the sheaths containing the daughter chromosomal coils to separate in the late telophase of the two-banded form. But when the processes of cell division, particularly the behavior of the centrioles, the direction of the achromatic figure, and the plane of cytoplasmic division, are considered the two-banded form is so different from *S. polygyra* that one wonders whether it is desirable to place it in the same genus with *S. polygyra*. On the basis of the chromosomes alone, no one familiar with hypermastigotes, especially those with spiral flagellar bands, would hesitate to separate *S. polygyra* generically from *Macrospironympha*, *Leptospironympha*, and *Spirotrichosoma*, the previously described forms with two spiral flagellar bands, for *S. polygyra* has two rod-shaped chromosomes, while *Macrospironympha*, *Leptospironympha*, and *Spirotrichosoma* each has a fairly large number of V-shaped chromosomes. But the two-banded form under consideration also has two rod-shaped chromosomes which, like those of *S. polygyra*, are clearly differentiated morphologically into a short one and a long one; and it, too, should be separated from the previously described genera with two spiral flagellar bands. It is plainly a question, then, of whether one should separate *S. polygyra* and the two-banded form generically on a basis of four clear-cut differences; number of flagellar bands, behavior of centrioles, direction plane of the achromatic figure, and the plane of cytoplasmic division. Aside from the number of flagellar bands, the differences are largely physiological and the present tendency is not to place organisms in separate genera on this basis. This leaves for consideration only the number of flagellar bands. If the extranuclear organelles other than the spiral flagellar bands of *S. polygyra* and the two-banded form were at all different, the two organisms should be separated generically, but it has been impossible, after using various fixatives and stains, to detect a single difference. Hence, I believe the two organisms are more closely related than the number of flagellar bands indicates. In other words, the number of flagellar bands in this case does not represent a fundamental difference, and since it doesn't, it should not be used to separate the two organisms generically.

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In view of the facts presented in the discussion above, the hypermastigote flagellate with two spiral flagellar bands in *Kalotermes* (*Paraneotermes* Light) *simplicicornis* (Banks) is placed in the genus *Spirotrichonympha* Grassi and Foa, 1911 and the species *bispira*, the species being new. In order to conserve space and to bring out more clearly the similarities and differences between *S. polygyra* and *S. bispira*, the morphology and processes of cell division in the two species are described in one paper.

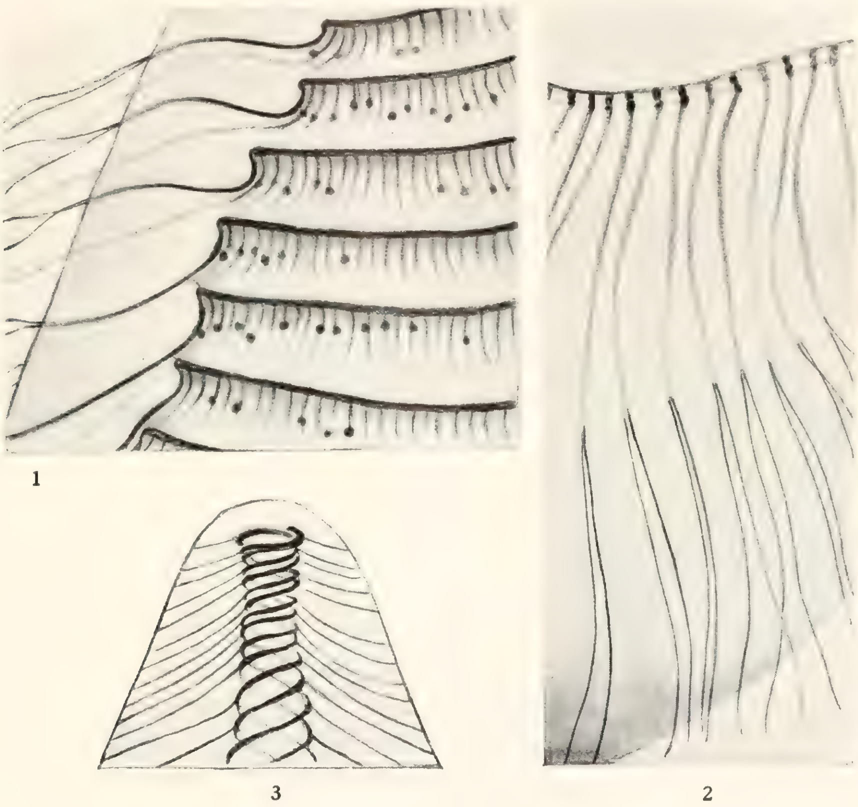
I am indebted to Professor S. F. Light for a generous supply of the termites used in this study, to Miss Jane Collier for technical and research assistance, to Miss Dorothy G. Harris for making the drawings, and to the Penrose Fund of the American Philosophical Society for financial assistance. It was necessary to make and study eleven hundred permanent preparations before obtaining the information presented.

SPIROTRICHONYMPHA POLYGYRA

Morphology

In ten individuals the body ranged in length from 63 to 112 microns with an average of 81; the width at the widest portion ranged from 25 to 60 microns with an average of 45; the distance of the greatest width from the anterior end averaged 56 microns; the distance from the anterior end to the nucleus averaged 21 microns; the transverse diameter of the nucleus averaged 11 microns; the distance from the posterior termination of the spiral flagellar bands to the posterior end of the body averaged 26 microns; the length of that portion of the body covered by the flagellar bands (the flagellated area) averaged 55 microns; the number of turns or spirals made by the flagellar bands averaged 45; the length of flagella from the surface of the body averaged 20 microns; the length of flagella from the basal granules to the surface of the body averaged 7 microns; the total length of flagella averaged 27 microns; the distance from the flagellar bands (straight line) to the surface of the body averaged 5 microns, and the width of a flagellar band is about 1 micron.

In the anterior end the spirals made by the flagellar bands are small and close together and do not vary much for a distance of 5 to 7 microns. Then they gradually become larger and farther apart until the mid-region of the body is reached. From this point on to their termination there is little variation in their diameter and in the distance between them (Fig. 1). They turn sharply at their point of origin in the anterior end, and this gives them a ring-like appearance when viewed vertically. When viewed laterally at their point of origin, they may be seen to originate in two groups, two in each group (Text-fig. *A*, 3). In other words, there are two points from which the bands grow out posteriorly, and two of the bands arising from each point lie side by side for a short distance. Sometimes, just before, but usually shortly after, cytoplasmic division the process of duplicating the flagellar bands begins. It cannot begin earlier, as in some related genera, because the four bands of the parent cell must unwind first and distribute two bands to each daughter, a process that is not completed in this flagellate until just before cytoplasmic division (Figs. 28–37). If the two new or daughter bands were to grow out before the unwinding of the parent bands was completed or nearly so, they would become entangled with the parent bands and



Text-fig. *A*. Details of portions of *Spirotrichonympha polygyra*. 1. Surface view of spiral flagellar bands. × 3000. 2. Flat surface of a band, showing rows of basal granules and the flagella that arise from the granules. × 4800. 3. Anterior end, showing how the flagellar bands begin to spiral. × 4800.

could never take their places besides them to form the interphase organism with four spiral flagellar bands.

When viewed laterally, depressions may be seen between the bands as shown in Text-fig. *A*, 1. In other words, the bands appear to be on ridges. This impression is probably produced by the ectoplasmic layer, which extends from the surface of the body to the bands, pushing inward between the bands. The flat surface of the flagellar bands does not lie parallel with the antero-posterior axis of the body as do the bands in *Macrospironympha*, but lies at an angle, the posterior margin of the band being nearer the surface of the body than the anterior, so that it is only when the bands are flattened that views of them as shown in Text-fig. *A*, 2 may be obtained.

The basal granules, from which the flagella arise, lie in rows antero-posteriorly directed across the flagellar bands, with two granules in each row (Text-fig. *A*, 2). The two flagella that arise from the granules in each row adhere to each other from their point of origin until they reach the surface of the body (Fig. 1, Text-fig. *A*, 1, 2). After leaving the band, the flagella extend posteriorly almost to the next band, from which point they turn somewhat abruptly and extend to the surface of the body, slanting posteriorly as shown in Fig. 1.

The large single axostyle is a hyaloid structure containing many fibres, extends from the anterior end through the central portion of the body, becomes wider as it approaches the nucleus which it surrounds, then smaller, enlarges again near the posterior end of the body, and after leaving the body tapers to a fine point. In the portion anterior to the nucleus the axostylar fibres are grouped close together, so that a few of them sometimes retain a small amount of stain in individuals suitably destained for nuclear details. This portion was mistaken for a centriole by Cupp (1930). By varying the destaining time, all degrees of destaining of the axostyle may be obtained, ranging from a heavily stained structure to one with no stain at all.

The so-called parabasals differ greatly in appearance from those of most genera of flagellates, but their reaction to fixatives and stains is the same, and I presume they should be regarded as parabasals. Instead of being long and slender, they are spherical and follow the spirals of the flagellar bands from a point near the posterior border of the nucleus to the termination of the bands (Fig. 1). They are probably attached to the bands since they follow them so regularly and since they retain their relationship to the bands when the bands and the body are broken up by drastic treatment; but it has been impossible to see a connection between them and the bands.

There are other structures, overlooked by Cupp and others who have studied *Spirotrichonympha*, which follow the flagellar bands just as closely as the parabasals and which are clearly connected to the bands by fine threads, each structure being connected by a single thread. These structures are considerably smaller than the parabasals, stain deeply following fixatives which do not render the parabasals stainable, and follow the bands a considerable distance anteriorly beyond the termination of the parabasals (Fig. 1). In every individual, they terminate at practically the same point, namely halfway between the mid-portion of the nucleus and the anterior end; and they extend posteriorly to the termination of the bands. But, unlike the parabasals, they lie closer together along the bands in the region of the nucleus and anterior to the nucleus than elsewhere. They, like the parabasals, probably arise from the bands, since various stages in their growth may be noted in new bands which are increasing in length posteriorly.

Mitosis

The chromosomes are duplicated in the late telophase (Fig. 15), and as a result of this each interphase nucleus contains four chromosomes (Figs. 3–7). Each chromosome contains a distinct coil within a sheath and is anchored to the anterior margin of the nuclear membrane by an intranuclear chromosomal fibre. These ends of the chromosomes are referred to as the anterior ends or as the attachment ends, while the opposite ends, which are attached either to a chromatin nucleolus or to portions of it, are referred to as the posterior ends. The portions of the chromatin nucleolus attached to the chromosomes vary considerably in size and shape (Figs. 3, 5); sometimes a single piece may be attached to all the chromosomes (Fig. 4); at other times a piece may be attached to each pair of chromosomes (Fig. 8). The daughter chromosomes are usually twisted around each other, the degree of twisting varying considerably in different nuclei. When all the chromosomes are attached to one nucleolus, they are twisted little if any, and in such nuclei the direction of the chromosomal coils may be followed plainly from one end of the chromosomes to the other (Fig. 4). It is also possible in many of the other nuclei where the chromosomes are each attached to a portion of the chromatin nucleolus and hence are permitted to twist considerably, to follow the coils clearly from the anterior to the posterior end of the chromosomes and to see that the coil sometimes reverses its direction in an individual chromosome (Fig. 3); and when each pair of daughter chromosomes is attached to a portion of the chromatin nucleolus, as illustrated in Fig. 8, the direction of the coils may be determined at a glance because they are so plain. A coil may reverse its direction at any point in a chromosome, and the coils of daughter chromosomes may be directed either alike or differently. Models of chromosomal coils, made of wires, show that the shifting of direction of coiling in a chromosome does not interfere with the coil splitting lengthwise, as it does in the telophase, and separating to become two individual coils of daughter chromosomes.

The first step in cell division is the separation of the spiral flagellar bands at their anterior ends, two of the bands moving in one direction and two in the other; and as they separate they unwind, a process that is not completed until shortly before cytoplasmic division (Figs. 28–37). Almost immediately after the bands begin to separate and unwind, the achromatic figure begins to form between the two groups of separating bands (Figs. 28, 29). In most dividing organisms the fibres of the achromatic figure, like the flagella, appear to arise directly from the flagellar bands about midway between the nuclei and the anterior ends of the cells, but in a few individuals—especially those distorted by drastic treatment—a fine, darkly staining line may be seen following one band of each group (of two bands). This line is the elongate centriole from which the fibres of the achromatic figure arise (Fig. 31). It should be noted here that in other genera of the Spirotrichonymphidae previously studied (Cleveland, Hall, Sanders, and Collier, 1934) two elongate centrioles follow the spiral flagellar bands from their point of origin at the anterior ends of the cells for a considerable distance posteriorly, but their posterior or distal ends, which produce the achromatic figure, are free of the bands. In *Pseudotrichonympha*, a hypermastigote without flagellar bands (Cleveland, 1935), the unusually long centrioles adhere to the inner margin of the rostral portion of the flagellated area, although this, comparatively speaking, is a short portion of the centrioles; in *Barbulanympha*, a hypermastigote with two, short flagellated areas at the anterior end instead of flagellar bands, the centrioles which are about 40 microns in length, adhere to the lamella underneath the flagellated areas for a distance of 6 to 10 microns; and in *Trichonympha*, another hypermastigote without flagellar bands, the posteriorly directed elongate centrioles are free except for their point of origin where they are fastened to the lamella underneath the flagellated area. Thus, there are all degrees of anchorage of the centrioles to another organelle, ranging from *Trichonympha*, *Joenia*, *Joenopsis*, and other genera, where the anchorage is slight, through *Barbulanympha*, *Urinympha*, *Rhynchonympha*, *Eucomonympha*, *Teranympha*, *Staurojoenina*, *Macrospironympha*, *Spirotrichosoma*, and *Leptospironympha* to *Spirotrichonympha*, where it is complete. Without this series, the situation in *Spirotrichonympha* would seem unusual and would be difficult to understand.

The manner in which the achromatic figure arises from the centrioles that follow two of the four flagellar bands is similar to that in other hypermastigotes: Astral rays arise from the distal end of each centriole, meet, join, overlap, and grow along one another to form the central spindle (Figs. 28–31). As more rays overlap, the central spindle becomes broader; and as the rays increase in length, it becomes longer. However, a point is reached shortly after nuclear division when the central spindle ceases to broaden, and it soon begins to narrow, because the daughter flagellar-band centriole complexes, to which its ends are anchored, move in opposite directions and thus pull apart the astral rays that formed the central spindle, the last-joined astral rays being the first ones to pull apart (Figs. 33, 35). Finally, the central spindle is pulled apart and disappears (Fig. 36).

The early central spindle lies a considerable distance anterior to the nucleus (Figs. 28–30), but, as it increases in size and the anterior ends of the developing daughter cells move in opposite directions, it is gradually moved toward the nucleus, finally stretching directly across it (Figs. 31, 32). The direction of the central spindle in *S. polygyra* is crosswise to the long axis of the body, while in *S. bispira*, as will be described presently, it extends parallel with the long axis. Shortly before the central spindle comes in contact with the ever intact nuclear membrane as a result of its moving posteriorly, some of the astral rays become extranuclear chromosomal fibres by making contact with the intranuclear chromosomal fibres anchored in the nuclear membrane, and as the central spindle increases in length, due to the centrioles from which it arises moving in opposite directions, these extranuclear chromosomal fibres begin to move the chromosomes toward the poles, one short and one long daughter chromosome moving toward each pole (Figs. 9, 10). In order to conserve space and since the same situation exists in *S. bispira*, where it is illustrated, the extranuclear chromosomal fibres have not been drawn; nor has the relation of the central spindle to the nucleus and chromosomes been shown in most instances. However, the position of the central spindle and of the extranuclear chromosomal fibres can be determined in the drawings of the nuclei by the direction of the intranuclear chromosomal fibres; the extranuclear chromosomal fibres extend from the intranuclear chromosomal fibres in the nuclear membrane in the same direction as the intranuclear chromosomal fibres (Figs. 9–12); and the direction of the extranuclear chromosomal fibres indicates the position of the central spindle, since they and the fibres of the central spindle arise from the distal ends of the centrioles. The nuclei (Figs. 3–15) are all mounted so that their anterior surfaces point toward the top of the page.

Just before the chromosomes begin their poleward movement, they become considerably shorter and the turns made by their spirals become broader. These stubby chromosomes persist until shortly after nuclear division (Figs. 9–12); then they gradually elongate and their coils and sheaths split lengthwise, thus producing one short and one long pair of chromosomes which persist through the interphase (Figs. 13–15).

SPIROTRICHONYMPHA BISPIRA SP. NOV.

Morphology

In ten individuals the body ranged in length from 59 to 102 microns with an average of 81; the width at the widest portion ranged from 32 to 48 microns with an average of 40; the distance of the greatest width from the anterior end averaged 57 microns; the distance from the anterior end to the nucleus averaged 21 microns; the transverse diameter of the nucleus averaged 10 microns; the distance from the posterior termination of the spiral flagellar bands to the posterior end of the body averaged 27 microns; the length of that portion of the body covered by the flagellar bands (the flagellated area) averaged 54 microns; the number of turns or spirals made by the flagellar bands averaged 34; the length of the flagella from the surface of the body averaged 20 microns; the length of the flagella from the basal granules to the surface of the body averaged 7 microns; the total length of the flagella averaged 27 microns; the distance from the flagellar bands (straight line) to the surface of the body averaged 5 microns, and the width of a flagellar band is about 1 micron.

It is clear from these measurements that *S. bispira* differs from *S. polygyra* neither in the size of the body nor in the size of the organelles. And the interphase organelles of the two organisms do not differ in appearance and number except for the number of bands and the enclosure of each pair of daughter chromosomal coils in a common sheath in *S. bispira*, differences already noted. There is, however, a notable difference in the number of turns made by the flagellar bands, those of *S. polygyra* making 45 and those of *S. bispira* making 34. Here is a fact which in itself indicates that the two-banded and four-banded forms are distinct organisms; for, if the forms with two bands making 34 turns developed two more bands and these new bands followed the old ones, as they would have to do, the number of turns would be 68. The bands of *S. bispira* are slightly farther apart, especially in the anterior end, than those of *S. polygyra* (Figs. 1, 2). The description given for the axostyle, flagella, parabasals, and other extranuclear organelles of *S. polygyra* takes care of these organelles in *S. bispira*, and we may proceed immediately with the description of mitosis.

Mitosis

The various processes connected in one way or another with mitosis and cell division in *S. bispira* are unusually interesting because they are so plain, because of their unique features, and because they differ decidedly from those of *S. polygyra*. There is one short and one long chromosomal sheath in the interphase nucleus and each sheath contains two chromosomal coils (Fig. 16). The division of the coils occurs in the late telophase (Fig. 27). Each coil is attached to the nuclear membrane by a fibre which is termed the intranuclear chromosomal fibre. Until the early anaphase, each coil is thus anchored to the anterior margin of the nuclear membrane (Figs. 16–20). (In all the drawings of nuclei the anterior margins are directed toward the top of the page.) The opposite or posterior ends of the chromosomes are all attached either to a single chromatin nucleolus or to portions of a chromatin nucleolus, as described in *S. polygyra*.

The first step in nuclear division is the division of the chromosomal sheath and the moving apart of the daughter chromosomal coils (Fig. 17). As the daughter chromosomes move apart, they frequently coil around each other (Figs. 17–19). This movement of the chromosomes is not poleward movement, but merely movement within the nucleus, and the daughters are probably interconnected during this period—although interconnections cannot always be seen—for later when they contract and straighten, just before poleward movement begins, interconnections may be seen plainly (Fig. 20); and these strands connecting the chromosomes are not broken until considerable progress in poleward movement has been made (Figs. 21–23). It should be noted here that poleward movement of the chromosomes is not to the right and left, as in *S. polygyra*, when the anterior end of the organism and the anterior surface of the nucleus are foremost, but is anterior and posterior (up and down). In other words, the chromosomes of *S. polygyra* move at right angles to the long axis of the cell, while those of *S. bispira* move parallel with the long axis. The reason for this will be clear when the development and function of the achromatic figure is explained.

So far as I know, there is no cell hitherto described where the behavior of the centrioles during the formation of the achromatic figure is the same as in *S. bispira*. There are two spiral flagellar bands in each interphase organism, but it has been impossible to determine whether a centriole follows one of these bands prior to the beginning of cell division, because during division a centriole usually follows one band so closely that the centriole and the band appear as one structure. In other words, the centriole cannot be distinguished from the band, and its presence during division, with the exception of a few instances, can be demonstrated only by its function. The elongate centriole which follows the band during division may persist as an elongate centriole during the interphase, just as the centrioles of *Barbulanympha* and many other genera persist; it may degenerate except for its anterior portion in the late telophase, just as the centrioles of *Pseudotrichonympha* do; or there may be one short and one long centriole in the interphase as in *Trichonympha*. At present, I see no way to determine which possibility is correct. But, irrespective of whether the posterior portion of one centriole which follows a band during division degenerates or persists during the interphase, the manner in which it functions during cell division may be demonstrated clearly.

In order to facilitate the explanation of the processes concerned with cell division, it seems desirable to name and label some of the structures involved in the processes. The two interphase flagellar bands which persist and do not change position during cell division are designated *parent flagellar bands* (*p.f.b.*). In most organisms a centriole follows one of these bands from its point of origin at the anterior end of the cell for a distance of 4 to 5 turns or spirals, and these two structures are designated *centriole parent flagellar band* (*c.p.f.b.*). Occasionally the centriole does not follow the band for more than two turns before it separates from it and continues independently; then it is labeled *centriole* (*c.*). The earliest stage in cell division is the growth of a new flagellar band from the point of origin of the parent flagellar bands posteriorly. This new flagellar band has a centriole which, in the early stages of development, follows it for its entire length, and where the two follow each other they are designated *centriole new flagellar band* (*c.n.f.b.*). This new band soon breaks loose from its point of origin, becomes free, and migrates posteriorly; then the end which at one time was connected to the parent flagellar bands at their point of origin in the anterior end of the cell is designated the *anterior end* (*a.e.*); the other end which, as development progresses, elongates beyond the point where the centriole terminates is designated the *elongating end* (*e.e.*). As this new band elongates posteriorly from its point of origin, it develops basal granules from which *flagella* (*f.*) grow out. *Astral rays* (*a.r.*) arise from the distal portion of the centriole following the parent flagellar band and soon meet those that arise from the same portion of the centriole that follows the new flagellar band; when they meet, they join, overlap, grow along one another and thus form the *central spindle* (*c.s.*). The centriole that follows one of the parent flagellar bands may be referred to as a *stationary centriole*, and the one that follows the new or free flagellar band as a *free centriole*, although it is not free in a sense because it is attached to the band. Similarly, the astral rays arising from the two centrioles may be referred to (Figs. 52, 53) as *astral rays* from *free centriole* (*a.r.f.c.*) and *astral rays* from *stationary centriole* (*a.r.s.c.*).

The formation of a new flagellar band—first attached at its point of origin, then free and migrating posteriorly while an achromatic figure is formed between it and one of the (stationary) parent flagellar bands—presents an interesting picture in the mechanics of cell division and renewal of extranuclear organelles. This migrating flagellar band, extending in length as it migrates, evidently has considerable difficulty in finding its way to the posterior end of the cell, for it is sometimes entirely outside the parent flagellar bands and sometimes partly outside and partly inside. Its movement is further handicapped because it is joined to one of the parent flagellar bands by the central spindle arising from its centriole and that of the parent flagellar band. But in most instances it eventually makes its way to the posterior end of the cell and, while so doing, picks up and carries with it two chromosomes, half of the nuclear membrane, and other nuclear materials. However, it sometimes fails to make this journey, and these instances are particularly interesting from the standpoint of the manner in which the achromatic figure is formed and its function in nuclear division. Two such instances are illustrated: in one (Fig. 52) the new band appears to be caught by the parent flagellar bands near its anterior end; in the other (Fig. 53) the new band appears to be lodged to the side and anterior to the parent flagellar bands. In the first instance, it is difficult to distinguish clearly astral rays arising from the centriole following the new band from the flagella that are arising from the new band, but the astral rays that are arising from the distal end of the centriole that follows one of the parent flagellar bands are long and plain and extend between the spirals of the parent bands to the nucleus (Fig. 52). In the second instance, astral rays may be seen clearly arising from the centriole following the new band; they extend inwardly and the flagella, for the most part, outwardly (Fig. 53). The astral rays that arise from the stationary centriole (*a.r.s.c.*) extend within the spirals to the nucleus. In neither instance is a central spindle formed, because the centrioles, from which the astral rays forming the central spindle arise, are so placed that the astral rays arising from one centriole cannot meet those arising from the other. In both instances the chromosomes have the appearance of telophase chromosomes, yet the pairs of daughter chromosomes have not moved apart because the proper formation and function of the achromatic figure was upset by the position of the free centriole which adheres to the improperly placed new flagellar band.

The fact that the free centriole in this organism assumes almost every conceivable position during its posterior migration is responsible for the great variation in the appearance of the achromatic figure. For example, the central spindle of the achromatic figure may extend from the free centriole to the stationary centriole entirely within the parent flagellar bands (Figs. 49–51, 55); it may be entirely outside the parent bands (Fig. 47); one portion of it may be inside and the other outside (Figs. 43, 48); or, as is more often the case, it may be partly outside and partly inside (Figs. 42, 44, 45). The end of the central spindle adjacent to the stationary centriole is fairly constant in shape when the centriole follows the parent flagellar band closely, because it assumes the shape of that portion of the centriole-band complex from which the fibres arise, and hence is cylindrical (Figs. 50, 51); but when this centriole does not follow the band closely, as occasionally happens, there is nothing to direct the fibres into a cylindrical position and they merely extend posteriorly from the distal portion of the centriole (Figs. 41, 42, 45). The shape of the other end of the central spindle depends entirely on the position of the new flagellar band and the centriole following it. If the centriole and band lie in almost a straight line, the adjacent end of the central spindle is flat or nearly so (Figs. 47, 51); if they lie somewhat in a cylindrical position, the adjacent end of the central spindle is somewhat cylindrical (Figs. 44, 50); if they are zigzag or wavy, so is the adjacent end of the central spindle (Figs. 43, 45, 46, 49). The position of the free band and centriole may also be such that only a few of the astral rays arising from the stationary centriole join those arising from the free centriole to form a central spindle outside the parent flagellar bands, while the other astral rays arising from the stationary centriole extend between the parent bands to the nucleus (Fig. 46). Many variations in the numbers of fibres that join or fail to join occur.

The rôle of those astral rays which, by becoming extranuclear chromosomal fibres, function in the movement of the chromosomes to the poles is plain. These rays are longer than those which in other hypermastigotes perform the same function. This is because the nucleus lies farther away from the centrioles, the point of origin of the rays, than in most cells. In other words, the astral rays become long before they reach the nucleus. In some instances these extranuclear chromosomal fibres may be followed all the way from their point of contact with the intranuclear chromosomal fibres in the nuclear membrane to the poles (Figs. 45, 55); in others they are lost among the fibres of the central spindle soon after they leave the nuclear membrane (Fig. 51). There can be no question regarding the fact that they carry one short and one long chromosome anteriorly and one short and one long one posteriorly, because, when the centrioles from which they arise are so situated in the cell that these fibres cannot reach the chromosomes, the chromosomes do not move apart and the nucleus does not divide.

The chromosomes are anchored to the anterior surface of the nuclear membrane until the anaphase, and if there were no interchromosomal connections between the daughters of the two pairs, two of the chromosomes would not move at all until the nuclear membrane began to elongate prior to pulling in two. The situation would be very simple, as is occasionally the case when the connections between the daughter chromosomes are broken in the prophase or metaphase: two chromosomes (a long one and a short one) move to the posterior surface of the nuclear membrane, the nuclear membrane elongates, pulls in two, and daughter nuclei are developed. But the more usual thing is for the connections between the daughter chromosomes to persist until the two chromosomes that are going to form a part of the posterior daughter nucleus are pulled to the posterior surface of the nuclear membrane (Figs. 21, 22). The connections between the posterior ends of the chromosomes are the last ones to break because the pull on the chromosomes is from their anterior ends. Incidentally, we have here an explanation of what produces the so-called equatorial plate stage in chromosomes; the pull on the daughter chromosomes from opposite directions (antero-posteriorly in this organism) causes them to take up a more or less central position within the nucleus. Variations of this stage, of which there are many in different organisms, result from the irregularity in the behavior of the connections between daughter chromosomes, those in some organisms breaking sooner than in others.

Cell Division

The type of cell division in *S. bispira* is so unusual that it deserves special attention. Shortly after the division of the nucleus and the disappearance of the achromatic figure, the new flagellar band, which now lies in the posterior end of the cell (Fig. 56), begins to arrange itself in the form of a spiral, the first portion to so arrange itself being the anterior end (Fig. 57). This is the end which in the early stages of mitosis lies adjacent to the anterior ends of the two parent flagellar bands (Figs. 38, 39), the end which does not elongate and which is labeled *a.e.* in the illustrations of the formation and development of the achromatic figure (Figs. 39–55). It now becomes the anterior end of the daughter cell which is developing at the posterior end of the parent cell (Figs. 56–59). This new band soon becomes arranged in spirals from the anterior to the posterior end (Fig. 58). Meanwhile, both the posterior and anterior developing daughter cells begin to form new axostyles (Figs. 56–58). Transverse cytoplasmic division soon occurs, producing two independent daughter cells; one, the anterior daughter, obtaining a nucleus and all the parent extranuclear organelles intact except the axostyle; the other, the posterior daughter, obtaining a nucleus, the new flagellar band, the centriole adhering to the new band, a small amount of the parent cytoplasm, and the extranuclear organelles such as flagella, parabasals, axostyles, etc. developed before cytoplasmic division. In other words, all the extranuclear organelles of the parent cell except the axostyle, which is resorbed, are carried over into the anterior daughter, and the posterior daughter gets the new organelles. The axostyle surrounds the nucleus in such a manner that the nucleus probably could not divide if this organelle remained intact. So far as I know, the extranuclear organelles of flagellates are either resorbed and each daughter develops a new set of organelles or the parent organelles are distributed among each daughter. *S. bispira* shows a radical departure from either procedure, and one wonders if this is not the beginning of a new stage of evolution in flagellates.

After cytoplasmic division, the posterior daughter, which is considerably smaller than the anterior one, continues the development of its extranuclear organelles. The axostyle increases in length and breadth and the flagellar band extends posteriorly, forming spirals as it does so, although the spirals for some time are not perfectly arranged (Fig. 59). They soon become arranged more or less perfectly (Fig. 60), and presently another flagellar band begins to develop from the point of origin of the existing or first new band. This band is referred to as the second new band. It extends posteriorly and soon takes its place along the first new band, so that the posterior daughter cell now has two spiral flagellar bands (Fig. 61). These bands increase in length and finally become as long as the parent bands were prior to the beginning of the processes of cell division. As the bands increase in length, they form more flagella and parabasals, the axostyle becomes grown, and the posterior daughter is now indistinguishable from the parent cell from which it originated (Fig. 2). Meanwhile, the axostyle of the anterior daughter completes its growth, and it, too, becomes indistinguishable from the parent cell from which it originated.

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EXPLANATION OF PLATES

The drawings were made with the aid of a camera lucida from material fixed either in Schaudinn's or Flemming's fluids and stained with hæmatoxylin.

PLATE 1

FIG. 1. *Spirotrichonympha polygyra.* Entire organism. Note four spiral flagellar bands. The flagella leave the bands in groups of two, and only two groups are shown from each band. The two adhering flagella extend posteriorly from their point of origin almost to the next band; then they turn and continue somewhat posteriorly to the surface of the body where they separate. The spherical parabasals follow the bands from a point just posterior to the nucleus to their termination. Other bodies, smaller than the parabasals, follow the bands from midway between the anterior end and nucleus to their termination. The axostyle extends from the anterior end around the nucleus to the posterior end of the body, terminating in a fine point slightly beyond the body. × 1600.

PLATE 2

FIG. 2. *Spirotrichonympha bispira* sp. nov. Entire organism. The extranuclear organelles differ from those of *S. polygyra* only in that there are two instead of four spiral flagellar bands. × 1600.

PLATE 3

Spirotrichonympha polygyra

The nuclei are mounted so that their anterior surfaces are directed toward the top of the plate.

FIG. 3. Interphase nucleus. There are four chromosomes. Each chromosome has a single coil lying within a sheath and is anchored to the anterior surface of the nuclear membrane by an intranuclear chromosomal fibre. The posterior end of each chromosome is attached to a portion of the chromatin nucleolus. There are two long and two short daughter chromosomes. The daughters of each group are somewhat coiled around each other. × 3800.

FIG. 4. Interphase nucleus. Both pairs of daughter chromosomes are attached to the chromatin nucleolus at their posterior ends; anteriorly they are attached to the nuclear membrane as explained in Fig. 3. × 3800.

FIG. 5. Interphase nucleus. Here the pairs of daughter chromosomes are considerably coiled around each other, the long pair lies over the short pair, and the posterior end of each chromosome is attached to a portion of the chromatin nucleolus. × 2400.

FIG. 6. Interphase nucleus more heavily stained than the three previous nuclei, so that other material in the nucleus besides the chromosomes stains and thus obscures slightly the arrangement of the chromosomes and their connections with portions of the chromatin nucleolus. × 3800.

FIG. 7. Interphase nucleus so heavily stained that chromosomal details cannot be seen. × 2400.

FIG. 8. Prophase nucleus. The daughter chromosomes are no longer coiled around each other. The coils within each chromosomal sheath are plain and their direction may be followed clearly. × 3800.

FIG. 9. Rather heavily stained nucleus. Each pair of chromosomes is short and stubby, the turns made by the coils within the sheaths are broader and closer together, and the daughter chromosomes are beginning to move apart. The chromosomal coils here and until the late telophase are not so distinct as in the interphase and prophase. The attachments between chromosomes and portions of the chromatin nucleolus will be lost presently, not to be renewed until the very late telophase. × 3800.

FIG. 10. The greatly shortened chromosomes have moved apart and a large one and a small one are preparing to line up on opposite sides of the nucleus. × 3800.

FIG. 11. Nucleus has elongated. One large and one small chromosome have been moved to the left side of the nucleus and the other two chromosomes are in the process of being lined up in the same manner. × 3800.

FIG. 12. The nuclear membrane has pulled in two and each daughter nucleus has a pair of dimorphic chromosomes. × 2400.

FIG. 13. Daughter telophase nucleus. Chromosomes are beginning to elongate and the coils within their sheaths are again distinct. × 2400.

FIG. 14. Daughter telophase nucleus. The chromosomal coils are in the process of duplicating themselves by longitudinal division. × 2400.

FIG. 15. Daughter telophase nucleus. The chromosomal coils have divided and the chromosomal sheaths have almost completed the process of longitudinal division. Connections are being made between chromosomes and portions of the chromatin nucleolus. × 2400.

PLATE 4

Spirotrichonympha bispira

The nuclei are mounted so that their anterior surfaces are directed toward the top of the plate.

FIG. 16. Interphase nucleus. Note a short and a long chromosomal sheath, each containing two chromosomal coils attached to the anterior margin of the nuclear membrane by an intranuclear chromosomal fibre. The posterior end of each sheath is attached to a portion of the chromatin nucleolus. × 3000.

FIG. 17. Prophase. The chromosomal sheaths are dividing longitudinally and each sheath contains a chromosomal coil attached anteriorly to the nuclear membrane and posteriorly to the chromatin nucleolus. The daughter chromosomes are coiled around each other. × 3000.

FIG. 18. Prophase. Daughter chromosomes arranged somewhat differently from preceding stage, the anterior attachments of the long pair having moved posteriorly, and each pair is attached posteriorly to a portion of the chromatin nucleolus. × 3000.

FIG. 19. Same as the two preceding stages except that each daughter chromosome is in the process of becoming attached to a single portion of the chromatin nucleolus. × 3000.

FIG. 20. Chromosomes have become short and stubby preparatory to poleward movement. × 4800.

FIG. 21. Early anaphase. The anterior ends of the long pair of chromosomes have made some progress in their poleward movement, one chromosome moving anteriorly, the other posteriorly. The short pair, as is sometimes the case, has been moved almost to the posterior end of the nucleus, although the chromosomes have not begun to separate. This was brought about by an extranuclear chromosomal fibre from the posterior pole (centriole) becoming attached to the intranuclear chromosomal fibre of the chromosome destined to go to the posterior pole. This fibre carried both chromosomes with it because the two chromosomes were connected. × 4800.

FIG. 22. A slightly later stage. The anterior ends of each pair of chromosomes have made considerable progress in their poleward movement, while the posterior ends are still connected and have not moved poleward. The short pair lies to the left and separation has progressed farther than in the pair which lies to the right. Note the evagination of the nuclear membrane at the four points where the intranuclear chromosomal fibres connect the chromosomes with the nuclear membrane. The evaginations are produced at these points by the pull of the extranuclear chromosomal fibres. × 3000.

FIG. 23. The chromosomes have turned completely around, a short one and a long one being directed toward each pole (anteriorly and posteriorly). Practically all of the connections between the daughter chromosomes have been broken. × 3000.

FIG. 24. The chromosomes have moved farther apart and the nucleus has begun to elongate. × 4800.

FIG. 25. The chromosomes have moved still farther apart and have begun to elongate. The nuclear membrane is constricting preparatory to being pulled in two. One intranuclear chromosomal fibre has been duplicated. × 3000.

FIG. 26. A later stage. Nucleus greatly elongated and will soon be pulled in two. Chromosomal coils in anterior pair are in the process of duplicating themselves by longitudinal division. × 3000.

FIG. 27. Posterior daughter telophase nucleus. The intranuclear chromosomal fibres and the chromosomal coils have been duplicated. The chromosomal sheaths, while slightly constricted, have not divided. × 4800.

PLATE 5

Spirotrichonympha polygyra

FIG. 28. Early stage in the separation of the four spiral flagellar bands into two groups. As the bands separate, they unwind. A centriole follows one band of each group from the anterior end to the posterior termination of the achromatic figure. Astral rays have arisen from the distal ends of each centriole; some of these rays have met and formed a portion of the central spindle; others, the posterior ones, are in the process of meeting to form more central spindle. × 1500.

FIG. 29. About the same stage as the previous one, but a different view of the two groups of separating and unwinding bands; anteriorly, the astral rays have met to form a portion of the central spindle; posteriorly, they have not met. × 1500.

FIG. 30. A slightly later stage in the separation of the bands and the formation of the central spindle portion of the achromatic figure. The astral rays have met both anteriorly and posteriorly to form the broad, flat central spindle. Note position of nucleus in this and in the two previous illustrations. × 1500.

FIG. 31. Later stage. Central spindle is longer, nearer the nucleus, bands are farther apart, and the unwinding of the bands has progressed considerably. × 1500.

FIG. 32. Still later stage. Central spindle is longer and lies over the nucleus. The bands which the centrioles follow are separated from the other bands. At the ends of the central spindle astral rays are arising from the centrioles and flagella are arising from the bands, but the flagella have been omitted and the astral rays have been omitted except those that joined to form the central spindle. × 1500.

FIG. 33. A much later stage. Nucleus has divided and daughter nuclei have moved a considerable distance apart; many of the astral rays have pulled apart so that the central spindle is now long and narrow. Note the manner in which the bands are unwinding. × 750.

FIG. 33 *A*. Detail of the anterior end of Fig. 33. Note the relation of the ends of the central spindle to the bands. × 1500.

PLATE 6

Spirotrichonympha polygyra

FIG. 34. Vertical view of a stage slightly later than Fig. 32. Note relation of central spindle to flagellar bands. The flagella are not drawn full length so as to avoid confusing them with the fibres of the central spindle. The astral rays that do not join to form the central spindle cannot be differentiated from flagella when the

two arise from the same point on the centrioles and flagellar bands. This is a six-banded individual. × 1500.

FIG. 35. An intermediate stage between Figs. 32 and 33 drawn to show that the ends of the central spindle are not flat but semicircular when the centrioles (and flagellar bands) from which the central spindle arises are not straight (as in Figs. 31, 32). In other words, the end of the central spindle has the same shape as the centriole (and band) from which the astral rays composing the central spindle arise. ×1500.

FIG. 36. A late stage in the unwinding of the flagellar bands. Achromatic figure has disappeared and daughter nuclei are far apart. New daughter axostyles are growing out and have extended a short distance beyond the nuclei posteriorly. × 1200.

FIG. 37. Elongate organism about to divide longitudinally. The unwinding of the parent bands is complete and new daughter bands will grow out presently. × 1200.

PLATE 7

Spirotrichonympha bispira

The flagella arising from the two parent or old bands and those arising from the new or free band are not drawn as long as they are. They are stopped where they leave the body.

Explanation of labels used in Plates 7–10.

a.e.—Anterior end (of new or free flagellar band).
a.r.—Astral rays.
a.r.f.c.—Astral rays from free centriole.
a.r.s.c.—Astral rays from stationary centriole.
c.—Centriole.
c.n.f.b.—Centriole of (or following) new flagellar band.
c.p.f.b.—Centriole of (or following) parent flagellar band.
c.s.—Central spindle.
e.e.—Elongating end (of new or free flagellar band).
f.—Flagella.
p.f.b.—Parent flagellar band.

FIG. 38. Anterior end of a cell in the earliest stage of division showing the two parent flagellar bands (*p.f.b.*), the new flagellar band and its centriole (*c.n.f.b.*), and flagella (*f.*), arising from the parent bands and the new band. The free centriole, which follows the new band from its point of origin in the anterior tip of the cell, has not begun to produce astral rays; nor has the stationary centriole which follows one of the parent bands. × 1500.

FIG. 39. A slightly later stage. The elongating end (*e.e.*) of the new band has begun to extend laterally, while the anterior end (*a.e.*) still remains at the anterior tip of the cell. × 1500.

FIG. 40. Later stage. The new band has begun to migrate posteriorly, its anterior end (*a.e.*) lying to the left and its elongating end (*e.e.*) to the right. The free centriole following the new band has given off astral rays which have joined some of those given off by the stationary centriole following one of the parent bands to form the very small, early central spindle (*c.s.*). In this instance, the centriole (*c.*) following one of the parent bands does not follow the band for its entire length, the distal portion being free. × 1500.

FIG. 41. The situation here is the same as in Fig. 40 except the new flagellar band is longer, has migrated farther posteriorly, the central spindle (*c.s.*) is longer and wider, and the stationary centriole is free of its band for a greater distance anteriorly. × 1500.

Fig. 42. Later stage. The new band, especially the elongating end (*e.e.*) has migrated farther posteriorly and now lies near the nucleus. The central spindle (*c.s.*) is longer. The distal portion of the stationary centriole (*c.*) is free of the band. × 1500.

Fig. 43. A considerably later stage in the development of the achromatic figure and the posterior migration of the new flagellar band. The central spindle (*c.s.*) is in two portions, one inside the parent flagellar bands and one outside. The portion inside the bands extends over the nucleus. No portion of the stationary centriole is free of the band. The shape of the end of the central spindle arising from the centriole following one of the parent bands is the same as the band and centriole from which it arises, semicircular. The other end of the central spindle is irregular in shape due to the position of the centriole from which it arises. Numerous astral rays (*a.r.*) extend toward the nucleus from the centriole that follows the new band. × 1500.

Fig. 44. Approximately the same stage as illustrated by Fig. 42, but different in appearance because of the position of the new band and its centriole. The end of the central spindle (*c.s.*) adjacent to the centriole of the new band is cylindrical or nearly so and no portion of the centriole of the parent flagellar band is free. × 1500.

Fig. 45. About the same stage as Fig. 44, but the achromatic figure presents a very different appearance because the new flagellar band and its centriole lie near the periphery of the cell and extend antero-posteriorly in almost a straight line, thus giving the posterior end of the central spindle a greatly flattened appearance. Note the length of the functioning portion of the centriole of the new band. × 1500.

Fig. 46. The achromatic figure here presents a strikingly different appearance from that of any of the previous illustrations, because many of the astral rays arising from the stationary centriole have extended posteriorly between the spirals of the parent flagellar bands instead of making their way toward the periphery, as a few of them have done, to join the astral rays arising from the free centriole (the one following the new flagellar band). Note astral rays extending from the free centriole toward the nucleus. When these rays join those arising from the other centriole and extending posteriorly within the spirals, another portion of the central spindle will be formed. This portion, like that illustrated in Fig. 43, will lie mostly within the flagellar bands. × 1500.

Plate 8

Spirotrichonympha bispira

The flagella arising from the two parents or old bands and those arising from the new or free band are not drawn as long as they are. They are stopped where they leave the body. See explanation of Plate 7 for meaning of labels employed.

Fig. 47. The central spindle (*c.s.*) lies almost entirely outside the parent flagellar bands. Its posterior portion, which arises from the centriole of the new flagellar band, is broad and flat because the new band extends in almost a straight line, while its anterior portion, which arises from the centriole of one of the parent flagellar bands, is semicircular. × 1500.

Fig. 48. Here there are two portions of the central spindle (*c.s.*), one inside and one outside the spirals of the flagellar bands. The centriole (*c.*) following one of the parent flagellar bands is free of the band for a short distance. × 1500.

Fig. 49. The central spindle (*c.s.*) lies within the spirals of the flagellar bands except for the posterior portion which is posterior to the bands. Astral rays arise from both the stationary and free centriole and extend toward the nucleus. Some of these rays have joined the intranuclear chromosomal fibres in the nuclear membrane and by so doing have become extranuclear chromosomal fibres which are pulling the chromosomes toward the poles, the centriole of the parent flagellar band (*c.p.f.b.*) and the centriole of the new flagellar band (*c.n.f.b.*). × 1500.

PLATE 9

Spirotrichonympha bispira

The flagella arising from the two parent or old bands and those arising from the new or free band are not drawn as long as they are. They are stopped where they leave the body. See explanation of Plate 7 for meaning of labels employed.

FIG. 50. Note the manner in which the astral rays composing the anterior half of the central spindle arise from the distal end of the centriole following one of the parent flagellar bands. The central spindle is almost cylindrical at this end because of the spiraling of the portion of the centriole from which it arises, while the opposite end is almost flat. There are probably astral rays arising from the centriole of the new band and extending toward the nucleus but, since they cannot be distinguished with certainty from the flagella which are numerous in this area, they are not drawn. × 1500.

FIG. 51. This central spindle is almost cylindrical anteriorly, flat posteriorly, and twisted in the mid-region. The astral rays that have become extranuclear chromosomal fibres by attaching themselves to the intranuclear chromosomal fibres in the nuclear membrane, as well as the free astral rays, are intermingled with the fibres of the central spindle and can scarcely be distinguished from them, thus presenting a situation closely resembling that of many metazoan mitoses. × 1500.

FIG. 52. In this cell the new flagellar band and its centriole failed to migrate posteriorly so that the astral rays arising from the free centriole (*a.r.f.c.*) could not meet and join those arising from the stationary centriole (*a.r.s.c.*) to form a central spindle. × 1500.

PLATE 10

Spirotrichonympha bispira

The flagella arising from the two parent or old bands and those arising from the new or free band, except in Fig. 54, are not drawn as long as they are. They are stopped where they leave the body. See explanation of Plate 7 for meaning of labels employed.

FIG. 53. Another cell where the free band and its centriole failed to migrate posteriorly and the astral rays arising from the free centriole (*a.r.f.c.*) could not meet and join those arising from the stationary centriole (*a.r.s.c.*) to form a central spindle. This would be a telophase if the proper formation of the achromatic figure had not been prevented by the position of the centrioles, for the chromosomes are telophasic in structure. × 1500.

FIG. 54. Many of the astral rays arising from the stationary centriole have failed to meet those arising from the free centriole and only a small central spindle connects the two centrioles. The movement of the chromosomes and the division of the nucleus have been upset. The flagella of the new band are drawn full length, but only one flagellum is shown beyond the point of bifurcation. × 1500.

FIG. 55. Nucleus is being pulled in two and the elongate central spindle is pulling apart. × 1500.

FIG. 56. Nucleus has divided, achromatic figure has disappeared, chromosomes have elongated, new axostyles are developing, and the new or free flagellar band has migrated to the extreme posterior end of the parent cell. × 1500.

PLATE 11

Spirotrichonympha bispira

The flagella arising from the two parent or old bands and those arising from the first and second new bands are not shown as long as they are. They are stopped where they leave the body.

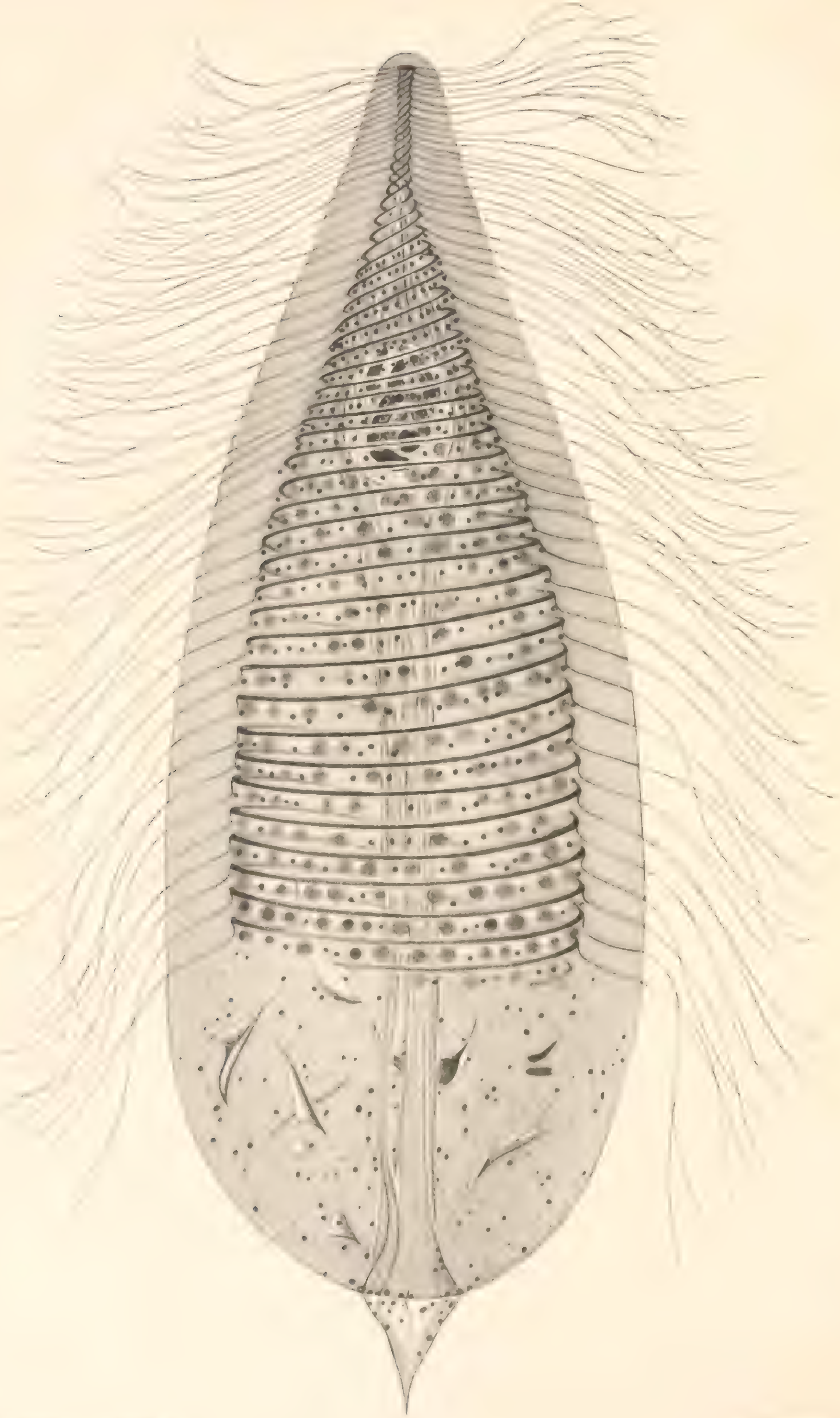
FIG. 57. The two parent flagellar bands, as in previous illustrations of cell division of *S. bispira*, remain intact. The new flagellar band which, although it has assumed various shapes in earlier stages, has not formed spirals, has now begun to spiral at its anterior end, which lies at the posterior end of the cell. The shape of the posterior end of the cell has changed, too. × 1500.

FIG. 58. Later stage. Cell is more elongate, posterior end is pointed more like the anterior end, and all of the new flagellar band has formed spirals. This stage is just before the transverse division of the cytoplasm to form two daughter cells; one, the anterior daughter, getting the two parent flagellar bands and their associated organelles; the other, the posterior daughter which is smaller, getting the new flagellar band and its associated organelles. Both daughters form new axostyles. × 1500.

FIG. 59. A posterior daughter cell after cytoplasmic division. The spirals of what was the new flagellar band before division of the body are little if any better arranged now. This organism has just separated from the anterior daughter. × 1500.

FIG. 60. A slightly later stage in the post-cytoplasmic development of a posterior daughter cell. The spirals of the flagellar band have perfected their arrangement. × 1500.

FIG. 61. A considerably later stage. A second new flagellar band has now appeared and is co-extensive with the first new band, the two forming spirals in the same manner as those of the parent cell previous to cell division. When these bands increase in length posteriorly (forming spirals as they do), the axostyle extends posteriorly, and the body increases in size, this cell will be like the parent which produced it. × 1500.



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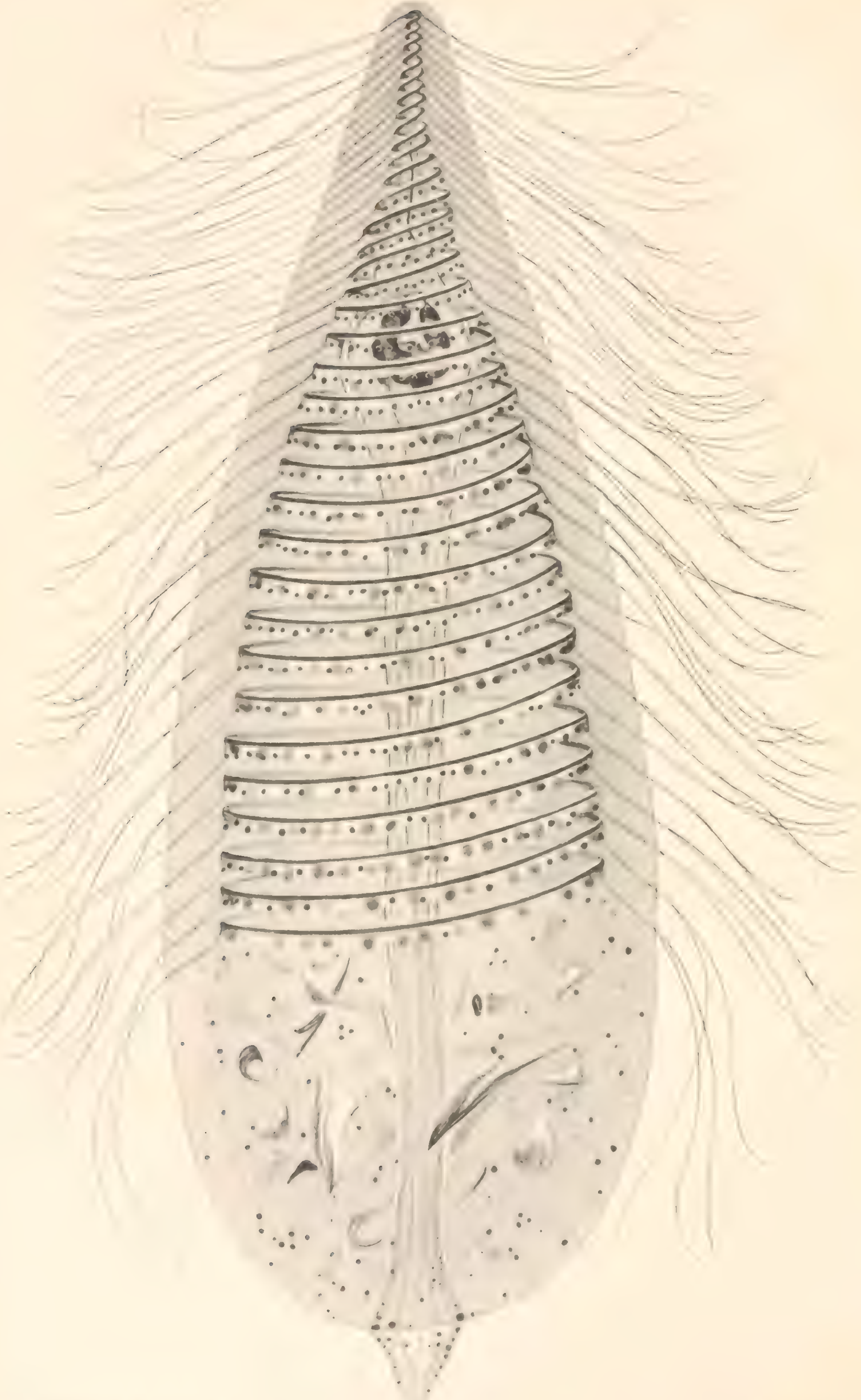

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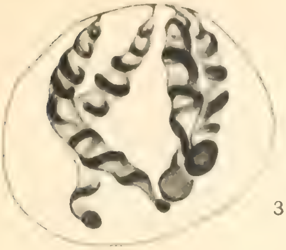
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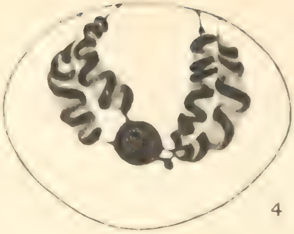
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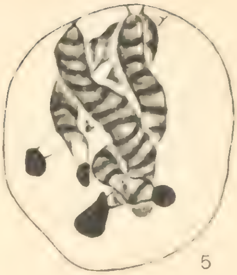
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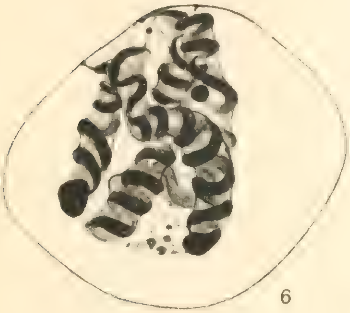
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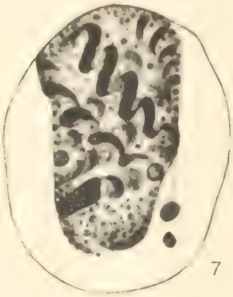
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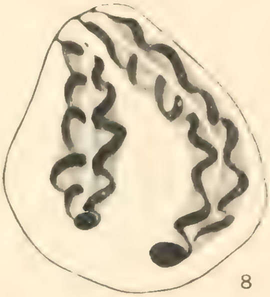
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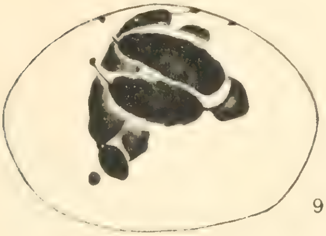
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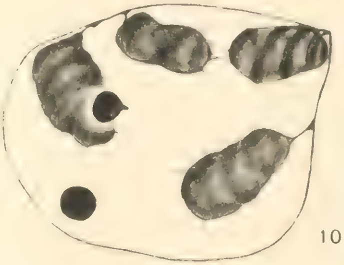
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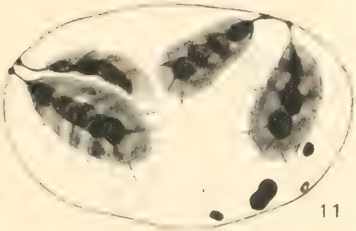
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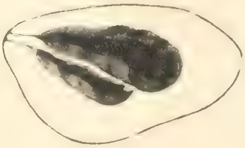
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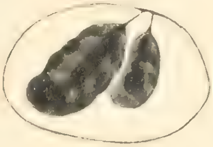

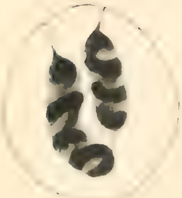
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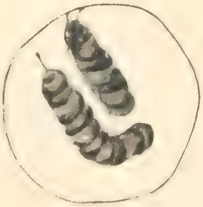
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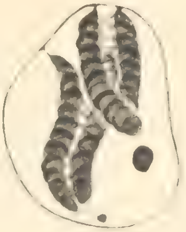
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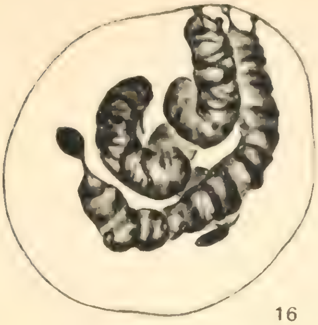
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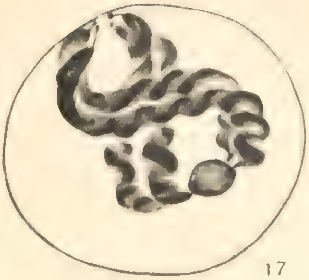
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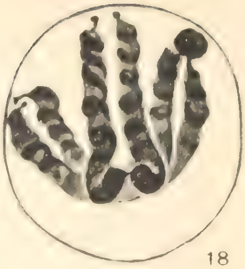
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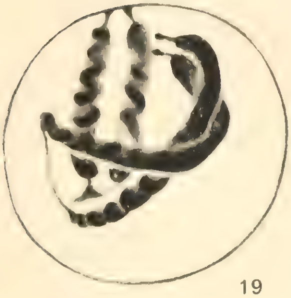
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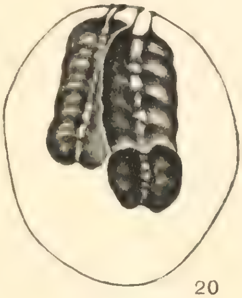
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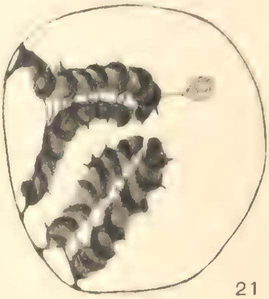
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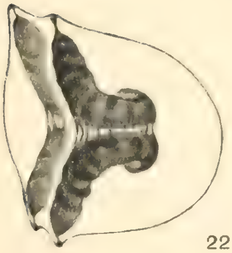
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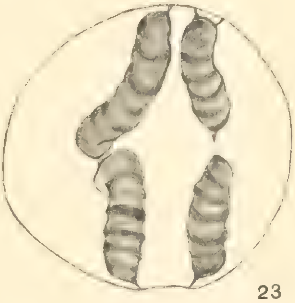
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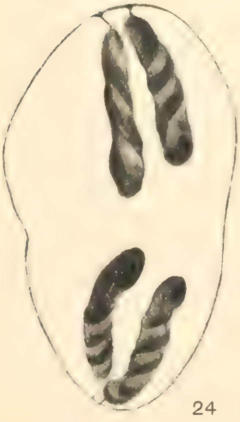
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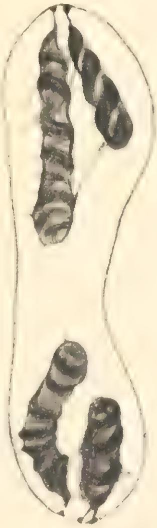
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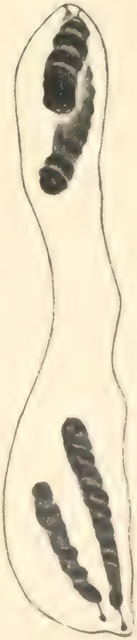
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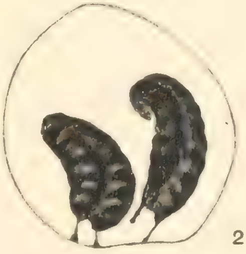
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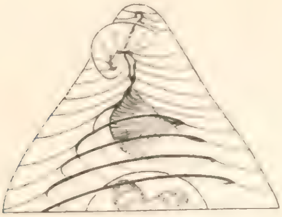

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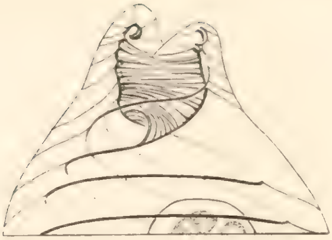

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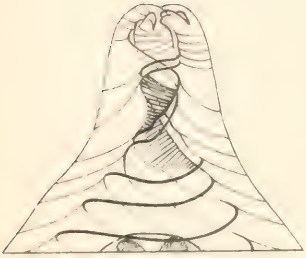

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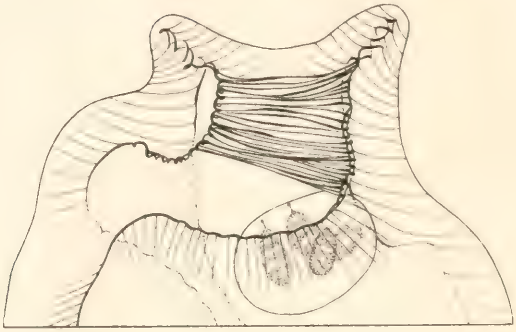

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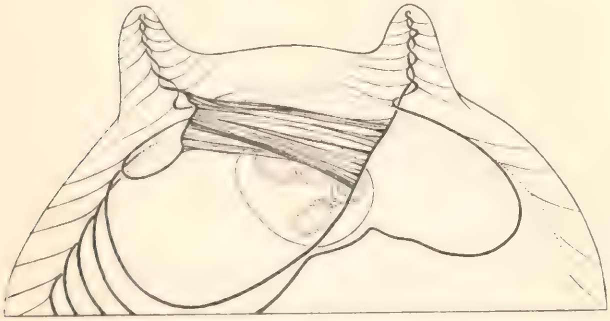

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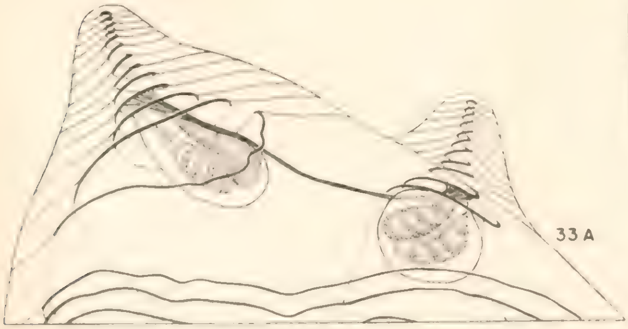

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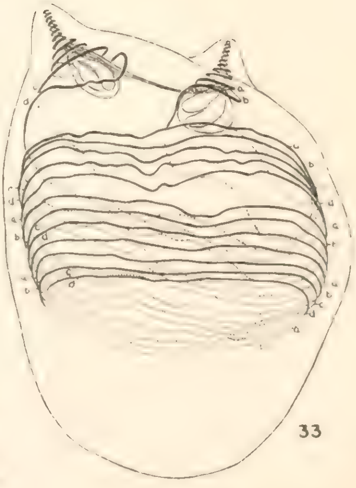

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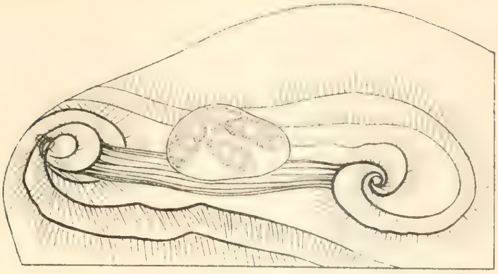

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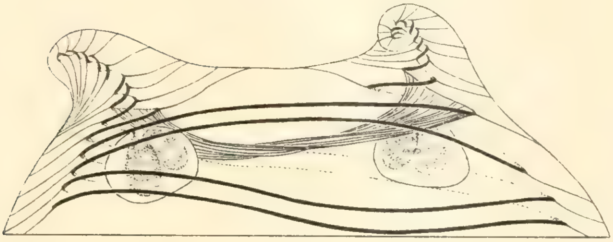

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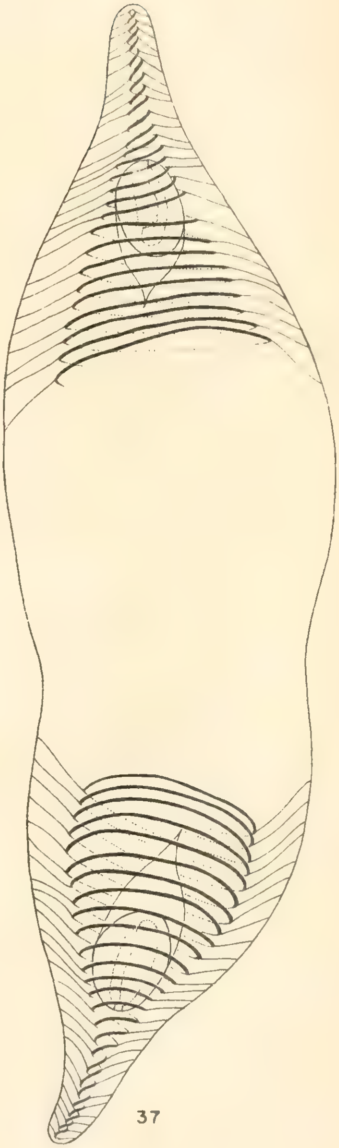

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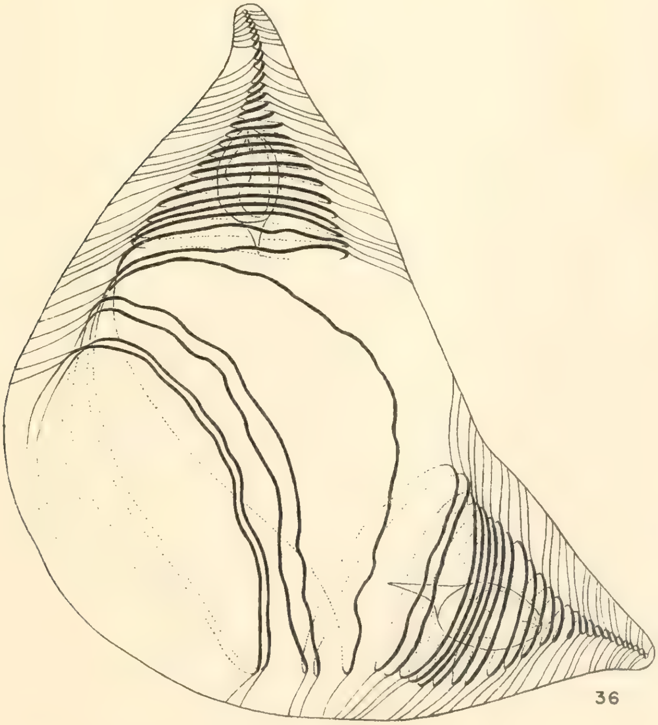

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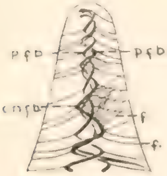
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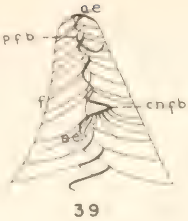
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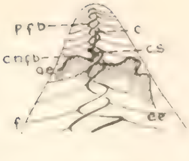
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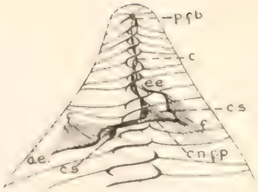
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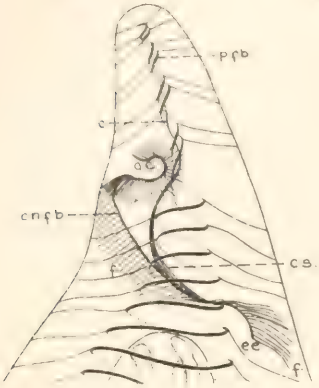
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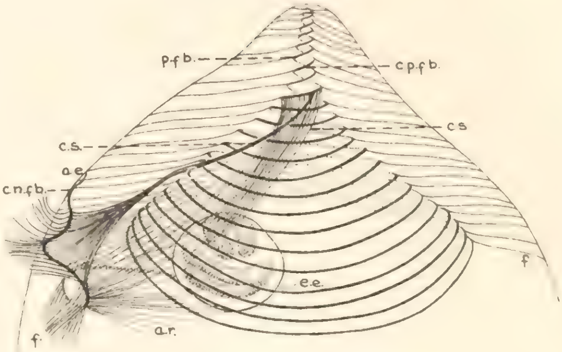
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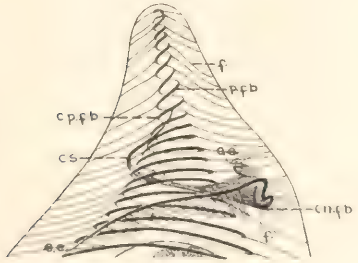
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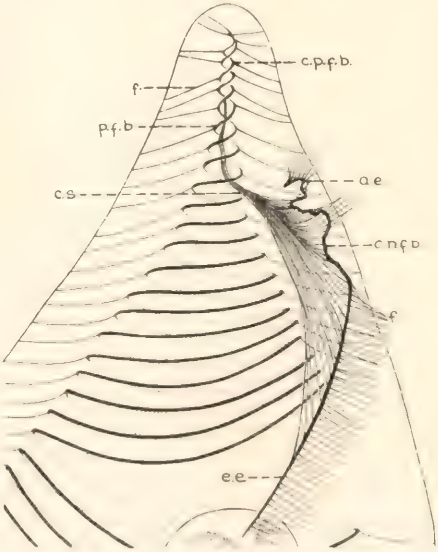
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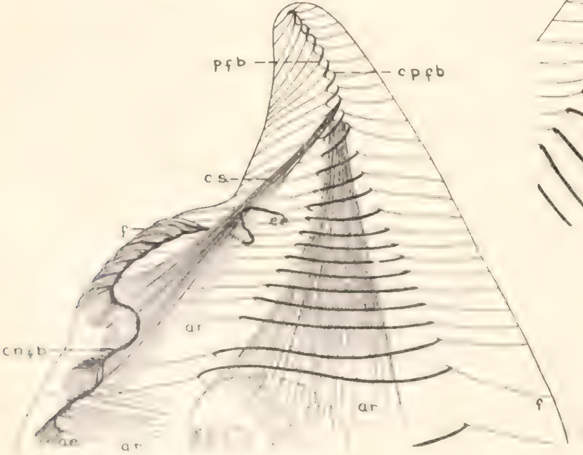
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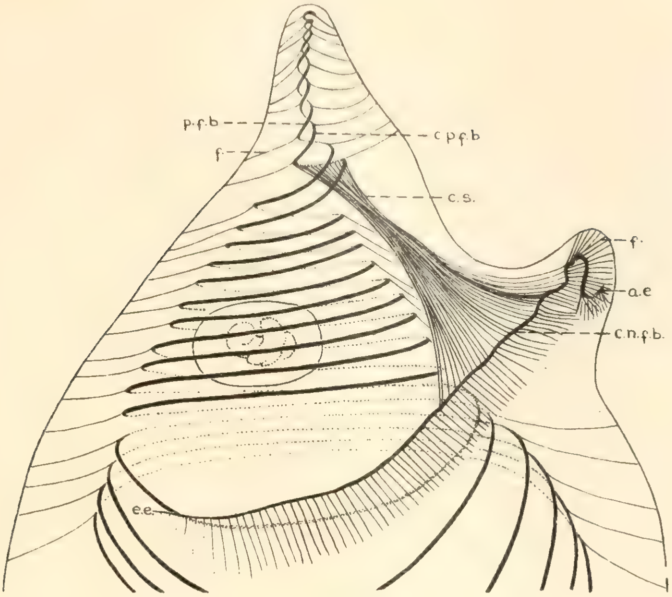
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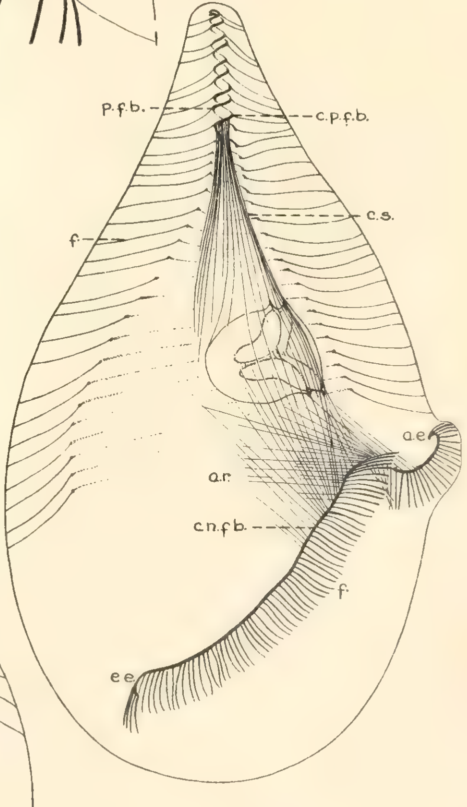
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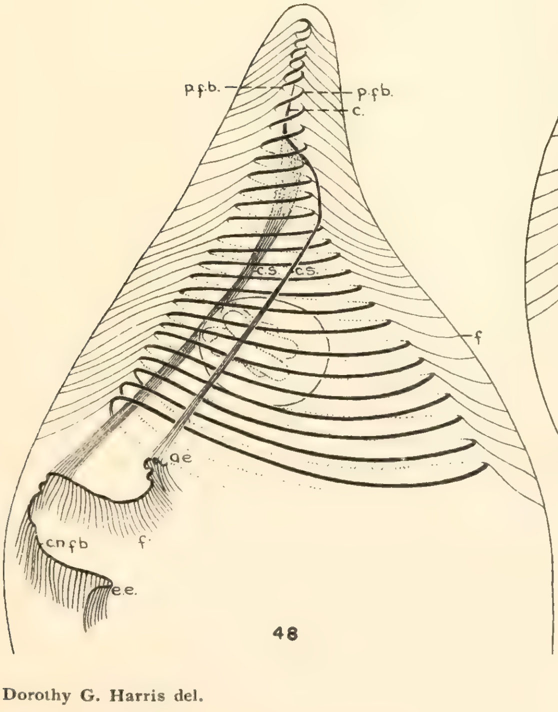
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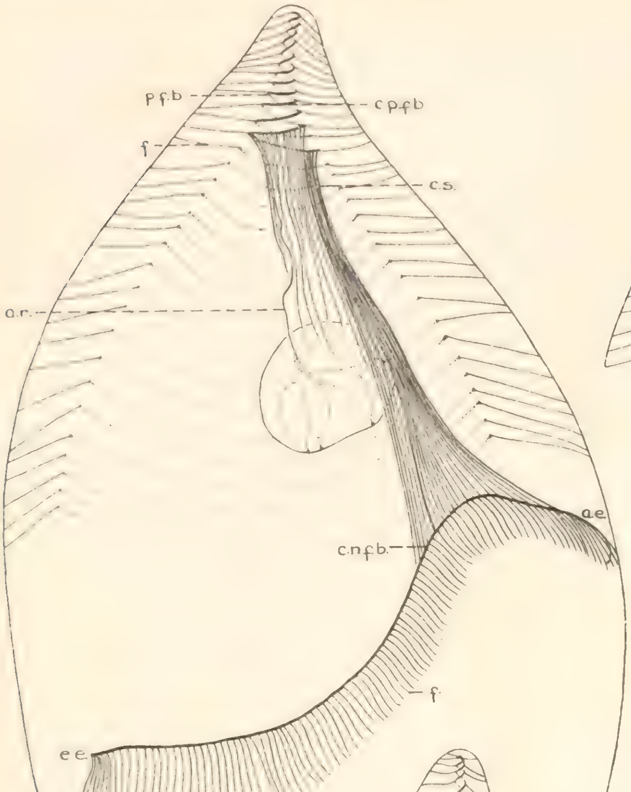
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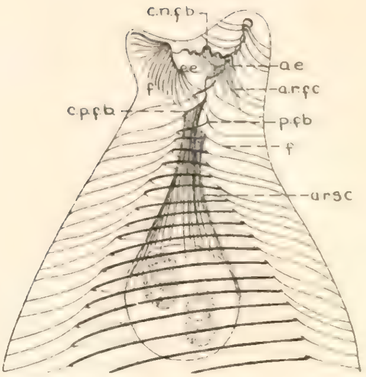
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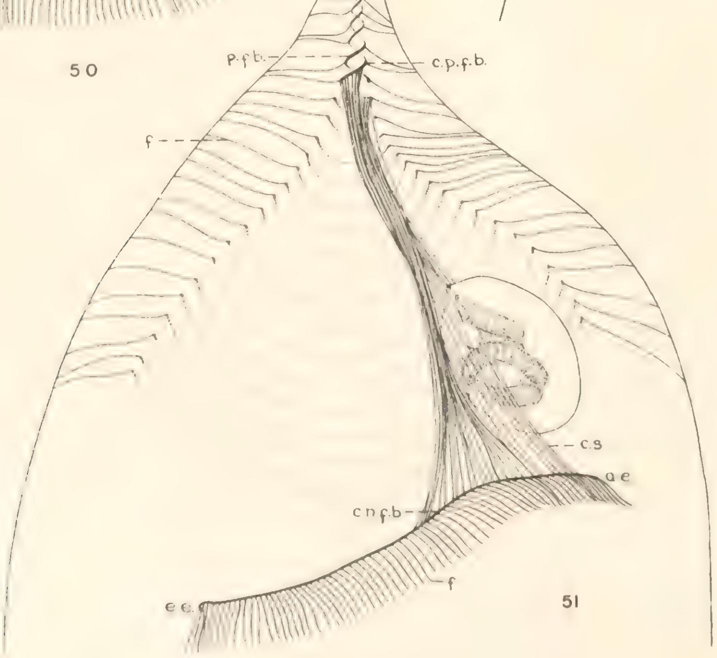
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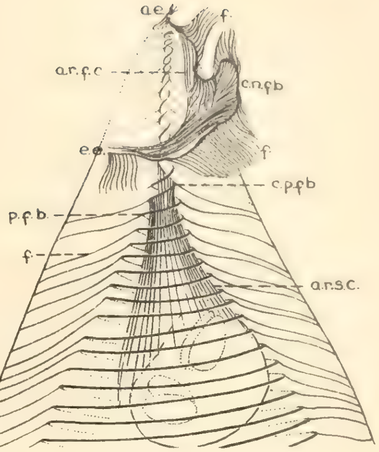
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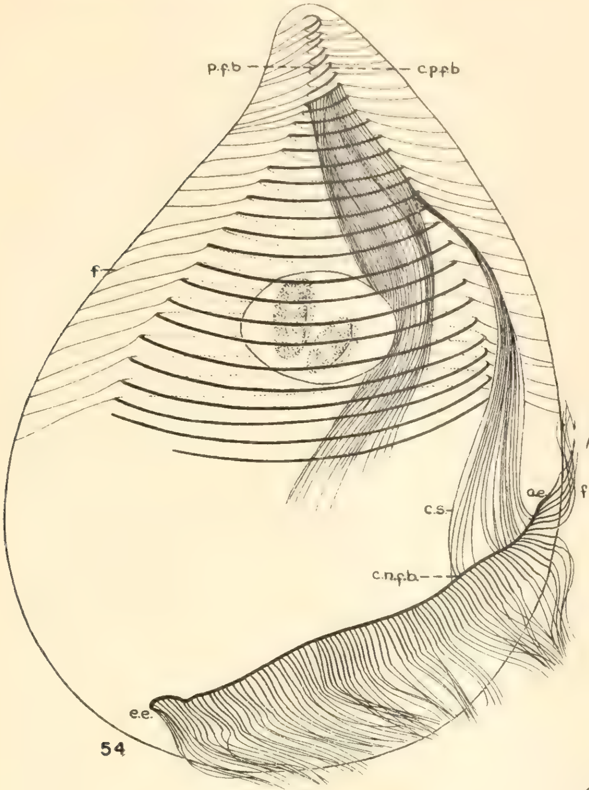
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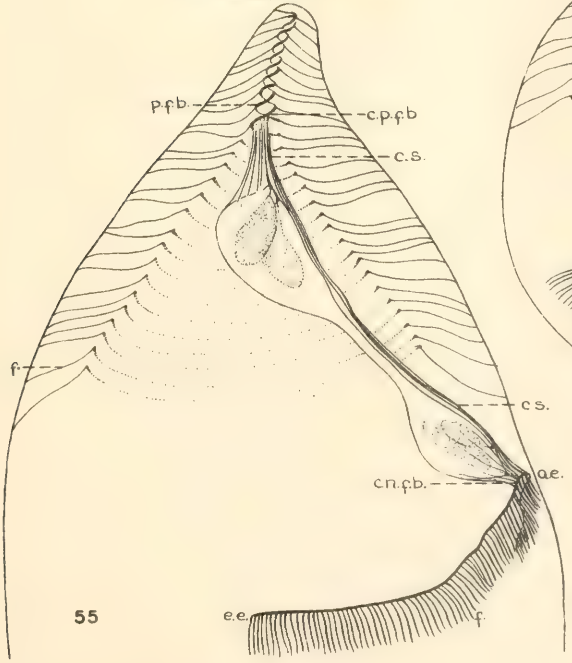
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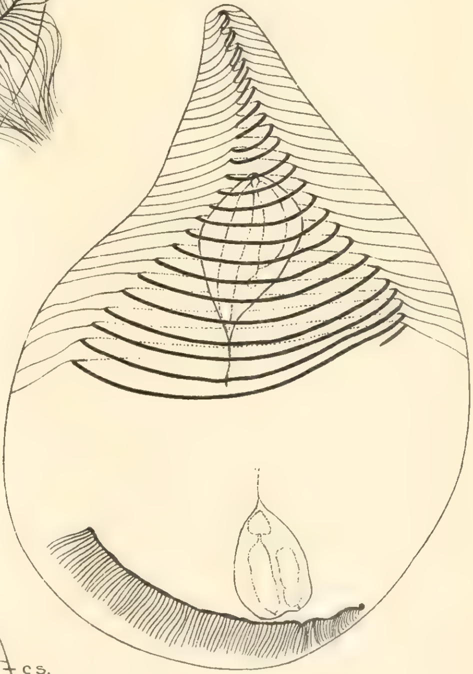

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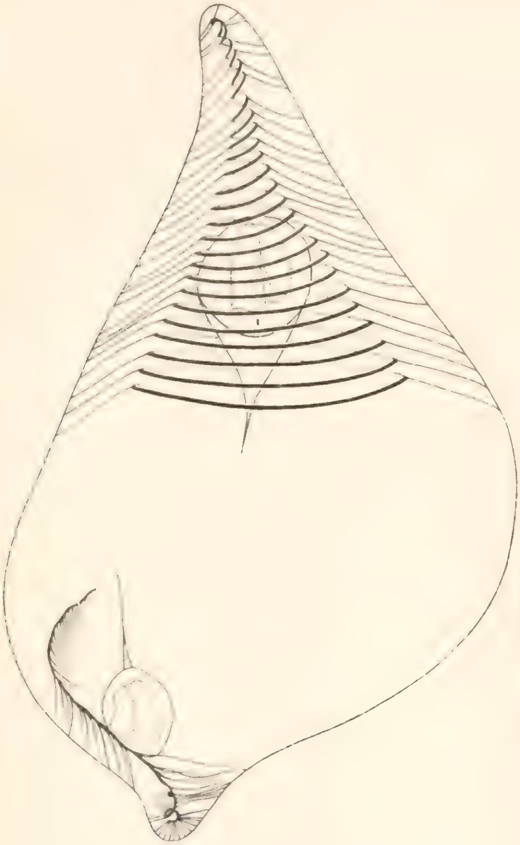

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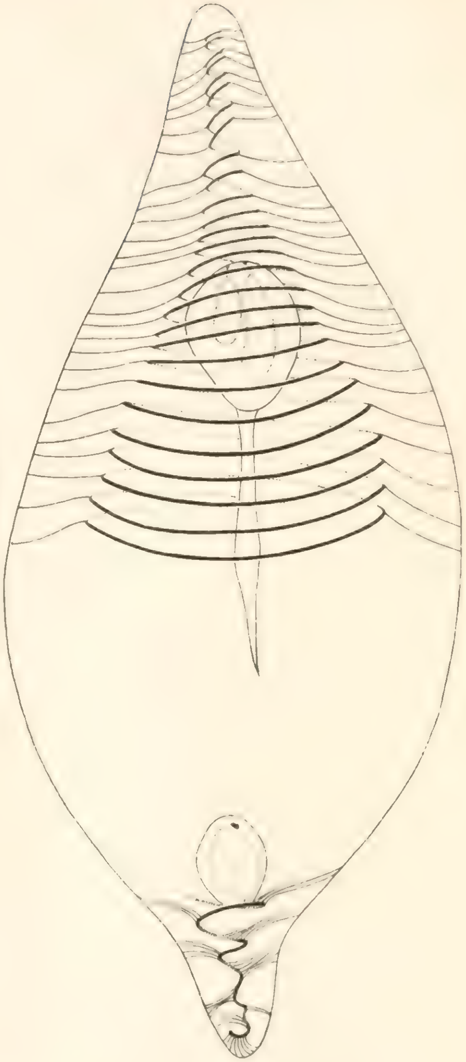

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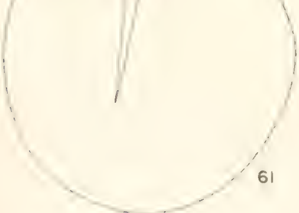

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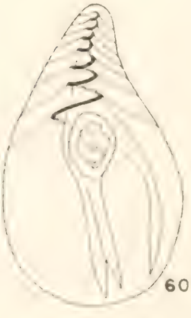

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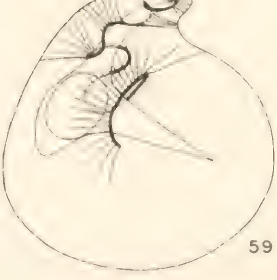

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ORIGIN AND DEVELOPMENT OF THE ACHROMATIC FIGURE

L. R. CLEVELAND

(*From the Biological Laboratories, Harvard University, Cambridge, Massachusetts*)

The question of the origin and nature of the achromatic figure has been debated for more than fifty years, and its existence has been seriously questioned many times, particularly during recent years. Several reasons have been advanced for regarding it as an artifact, the principal one being the inability to demonstrate clearly its origin, development, and function in living cells.

I have studied the achromatic figure in twenty genera of hypermastigote and ten genera of polymastigote flagellates. These organisms furnish a diversity of favorable material. In some of them, there is no question regarding the manner in which the achromatic figure is formed from the centrioles and the reality of the fibres composing it; in others, the development of the achromatic figure is not so plain, and can be determined with certainty only in view of their relation to those organisms where the process is unmistakably clear. There are gradations between the two groups. In several genera, the development of the achromatic figure has been followed in living cells from its very beginning to its disappearance in the late telophase; and the picture presented is exactly the same as in fixed cells.

In one genus, *Barbulanympha*, considerable time has been devoted to the origin and development of the achromatic figure when more than two centrioles are concerned in the process, and when only one is concerned; because the process is so very plain under these conditions that no one can question it. In a previous paper (Cleveland, Hall, Sanders, and Collier, 1934. *Mem. Am. Acad. Arts and Sci.*, **17**: 185) *Barbulanympha* was described, and an account was given of the manner in which the two elongate, interphase centrioles produce daughter or new centrioles like themselves, a process which need not be considered here; but it is desirable, before considering the achromatic figure when an abnormal number of centrioles are present, to consider it when two are present.

The centrioles vary in length in the four species of *Barbulanympha* from 15 to 30 microns; in the unstained living cell they are of a dense hyaline nature, and may be differentiated easily from other cellular contents; when fixed in Schaudinn's fluid and stained with Heidenhain's hæmatoxylin, they stain and destain in about the same manner as chromatin; they are joined at their anterior ends by a desmose; and their distal ends, which lie 20 to 30 microns apart, are free (Fig. 1).

The distal end of each centriole is surrounded by a spherical centrosome, which is 4 to 6 microns in diameter, and which moves with the centriole whenever the latter is moved, either by the natural movement of the cell or by mechanical manipulation. When the nucleus and centrioles are suitably destained for study, the centrocomes retain little or no stain.

In the interphase cell, there are no fibres extending from either sentriole (Fig. 1). In the prophase, astral rays begin to grow out from the distal end of each centriole (Fig. 2). At first, the rays arising from one centriole are a considerable distance from those arising from the other; but, as they increase in length, the two sets of astral rays soon meet (Fig. 3), and, as they meet, the individual rays or fibres join, grow along one another and overlap to form the early central spindle portion of the achromatic figure (Fig. 4). That the astral rays arise from the centriole instead of the centrosome may be shown by observation of living cells; the rays may be traced through the centrosome to the centriole. The same may be demonstrated also in fixed cells. And further proof that the centrosome plays no part in the production of the astral rays may be obtained from a study of several other genera with elongate centrioles similar to those of *Barbulanympha* but with no centrosomes surrounding their distal ends; in these, the connection of the astral rays to the centrioles may be seen at a glance. Those genera with centrosomes surrounding the distal ends of their centrioles form cylindrical central spindles, while those with no centrosomes form flat ones; and thus the rôle of the centrosome in directing the astral rays that form the central spindle may be seen.

As the astral rays continue to increase in length, more of those arising from one centriole meet those arising from the other, and the central spindle becomes larger; and, at the same time, the central spindle increases in length, because the centrioles from which it arises move in opposite directions (Fig. 5). Those astral rays which do not participate in the formation of the central spindle, because they do not meet and overlap, also increase in length; at the same time, more astral rays arise from each centriole, so that the individual rays vary greatly in length; and it is not long after the central spindle is formed before some of the astral rays which do not participate in its formation become extranuclear chromosomal fibres by joining the intranuclear chromosomal fibres, the fibres which anchor the chromosomes to the ever intact nuclear membrane (Fig. 6). Thus, the achromatic figure consists of astral rays which are joined together to form the central spindle, astral rays which are connected to the chromosomes and are responsible for their movement to the poles, and astral rays which merely radiate in the cytoplasm and, so far as known, perform no function. All of these fibres appear alike structurally, and the only justification for naming them is their functional differences.

When three centrioles are present in the interphase (Fig. 7), the type of achromatic figure produced by them during cell division varies considerably, depending on their position. If the distal ends of two of the centrioles lie fairly close together, as illustrated in Fig. 8, two long central spindles and one short one are produced. In this instance, the centriole which lies somewhat apart functions jointly with each of the other centrioles in the production of the two long central spindles; and the two centrioles which lie fairly close together function jointly in the production of a short central spindle between them. In other words, the astral rays arising from the centriole which lies more or less alone, and which may be termed the remote centriole, meet, join, and overlap those arising from the two centrioles which lie fairly close together, and thus the remote centriole functions as much again as each of the other centrioles in the formation of the two long central spindles, because it supplies as many astral rays for central spindle production as both of the other centrioles combined. If, however, as is frequently seen, two centrioles lie adjacent and one apart, the three centrioles function in the production of one central spindle, which has the same appearance as when only two centrioles are functioning in the production of a central spindle (for example, as in Fig. 5). The achromatic figure appears exactly as that of Fig. 8 would appear if the two centrioles on the right were pulled together so that one centrosome touched the other.

If the distal ends of the three centrioles are more or less equidistant, i.e., at the apices of an equilateral triangle, each centriole functions in approximately the same manner in the production of the achromatic figure; three central spindles, more or less equal in size, are produced, and each centriole functions in conjunction with two other centrioles in their formation (Fig. 10). If, on the other hand, the three centrioles are not equidistant, but lie at the apices of a triangle which is wider on one side, only two central spindles are produced—at least at first; a third may be formed later, as the astral rays become longer, if the centrioles between which a central spindle failed to form earlier do not lie too far apart (Fig. 9). Three is the maximum number of central spindles that can be produced from three centrioles.

Many cells with four centrioles have been studied. The four interphase centrioles may all be mature, i.e., fully grown, or some of them may be in the process of development, as shown in Fig. 11; but they do not function until growth is completed; and when they function, the type of achromatic figure produced by them depends on their position. If they lie side by side in groups of two, one central spindle is produced, although all four centrioles participate in its formation; if they lie at the four corners of a square, six central spindles are produced; if they lie at the corners of a rectangle, four central spindles are usually produced; if they lie fairly close together, but not adjacent, in groups of two, two central spindles are usually produced in the early stages of cell division, and in the later stages, if the centrioles of each group move apart, a central spindle is formed between the centrioles of each group. Sometimes, the four centrioles lie in the same plane in groups of two in such a manner that a criss-crossing occurs in the astral rays forming the central spindles. Figure 13 illustrates an example of this. Here astral rays arising from the upper centriole on the right have joined astral rays arising from both of the centrioles on the left, so that this centriole is connected by a central spindle to each of the centrioles on the left; and the astral rays arising from the lower centriole on the right have joined astral rays arising from both of the centrioles on the left, so that this centriole is also connected by a central spindle to each of the centrioles on the left. In brief, each of the four centrioles is connected to two other centrioles by a central spindle. Would such a series of connections occur as a result of "lines of force"? Other instances have been seen where, for example, the lower centriole on the right was connected to each centriole on the left by a central spindle, while the upper centriole on the right was connected only to the upper centriole on the left by a central spindle. And other variations have been seen in the criss-cross central spindle interconnections of centrioles.

When five non-adjacent centrioles are present, eight central spindles could be formed by an overlapping of the astral rays arising from them, although six, as shown in Fig. 12, is the usual number. Here each of the two upper centrioles is functioning in the formation of three central spindles; the lower right and left centrioles are each functioning in the formation of two central spindles; and the lower middle centriole is functioning in the formation of four central spindles, most of its astral rays being used to form central spindles.

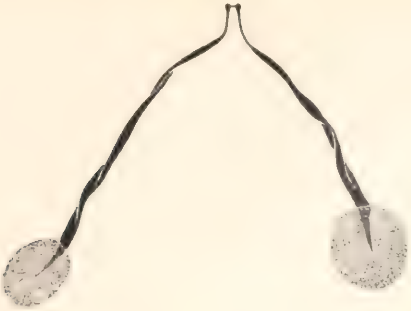
Many examples of a still larger number of centrioles functioning in the production of multiple achromatic figures have been studied, but only one has been illustrated; this is Fig. 16 where fifteen centrioles are present in order to avoid confusion only the centrosomes are drawn).

Here there is great variation in the length and width of the central spindles, resulting from differences in the time of their formation and in the number of fibres composing them. There are also many instances of the criss-crossing of the fibres of the central spindles; instead of the astral rays arising from one centriole joining those from another, they join those from several centrioles. Few of the central spindles are straight, that is, extend directly from one centriole to another; and it is difficult, owing to their curvation and the manner in which they are crowded together, to determine the number present.

When two centrioles are present but are so widely separated that the astral rays arising from one cannot meet those arising from the other, the central spindle portion of the achromatic figure is not formed (Fig. 14). Similarly, when only one centriole is present, the development of the achromatic figure is incomplete; many astral rays extend to and around the nucleus, but they do not form a central spindle (Fig. 15).

In a few instances where several interphase centrioles are present, decidedly abnormal conditions have been seen; the formation of centrosomes surrounding the distal ends of the centrioles is either completely or partially upset, and strands, varying in size from a little larger than astral rays to half the size of the centrioles, extend posteriorly from the centrioles (Fig. 17). Just what these strands are is not known. They can scarcely be regarded as astral rays, or even a premature aberrant attempt to produce an achromatic figure; but they may be secondary extensions from the centrioles.

I am indebted to the Penrose Fund of the American Philosophical Society for financial assistance.



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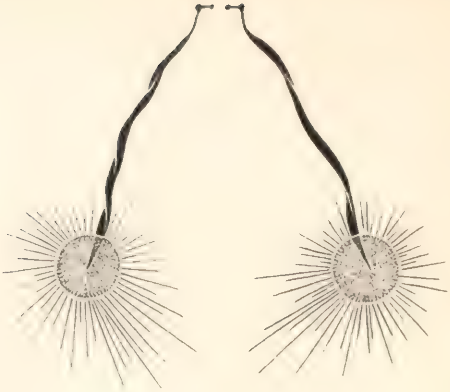

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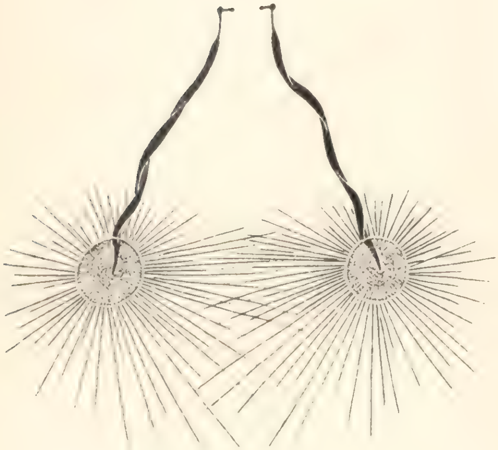

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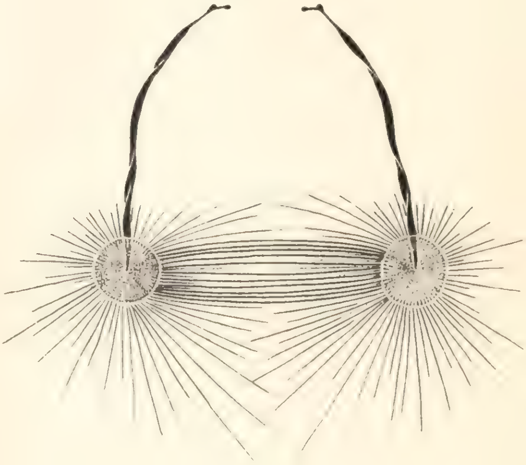

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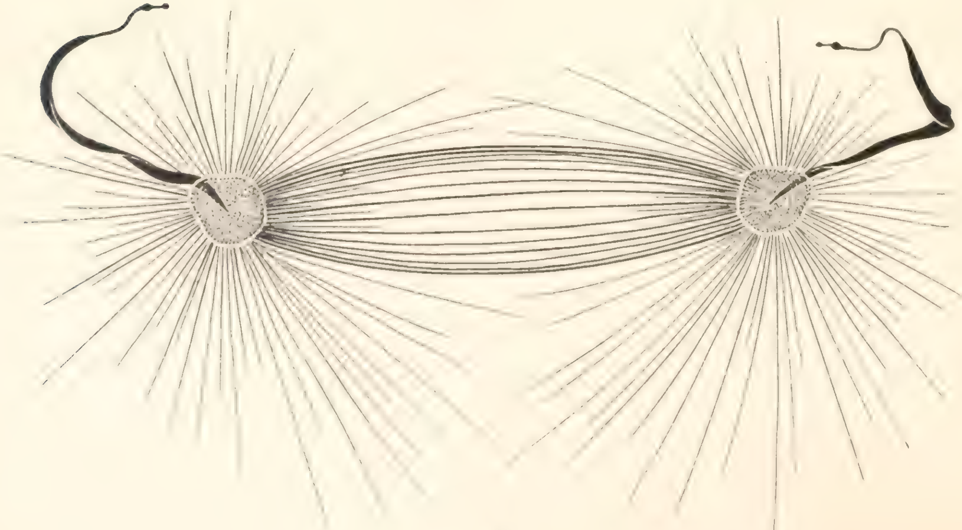

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EXPLANATION OF PLATES

All figures were drawn with the aid of a camera lucida from material fixed in Schaudinn's fluid and stained with Heidenhain's hæmatoxylin. The organism is *Barbulanympha*.

PLATE 1

FIG. 1. The two elongate interphase centrioles with centrosomes surrounding their distal ends. × 1400.

FIG. 2. Prophase centrioles with astral rays arising from their distal ends. × 1400.

FIG. 3. The astral rays arising from one centriole are meeting those arising from the other, preparatory to the formation of the central spindle. × 1400.

FIG. 4. The astral rays have met, joined, and grown along one another to form the early central spindle. × 1400.

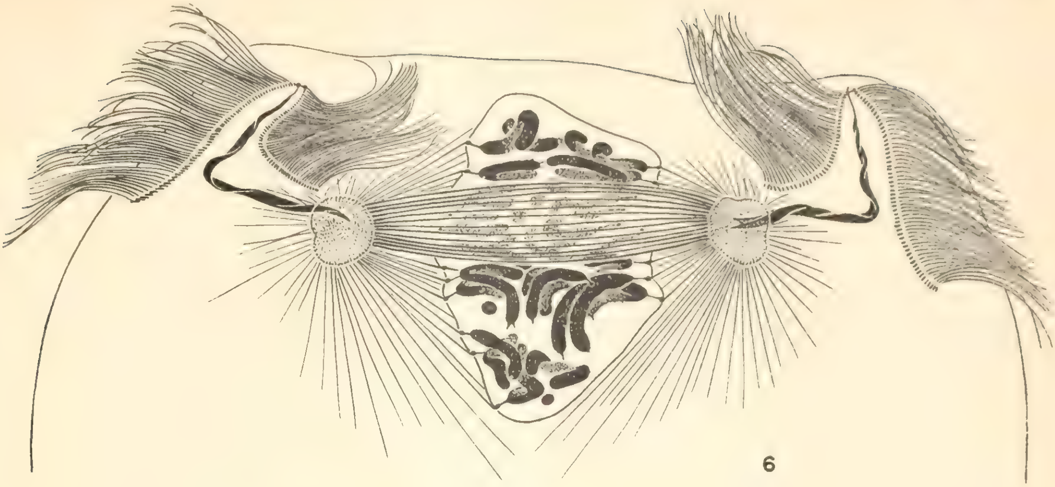
FIG. 5. More astral rays have joined and the formation of the central spindle is complete. × 1400.

PLATE 2

FIG. 6. Longitudinal section of anterior end of cell showing the interrelation of flagellated areas, centrioles, achromatic figure, and chromosomes. Some of the astral rays which do not join and overlap in the formation of the central spindle have become extranuclear chromosomal fibres by connecting with the intranuclear chromosomal fibres in the nuclear membrane. × 1400.

FIG. 7. Three interphase centrioles and centrosomes. × 1400.

FIG. 8. The type of achromatic figure produced by three centrioles when they lie in this position. × 1400.



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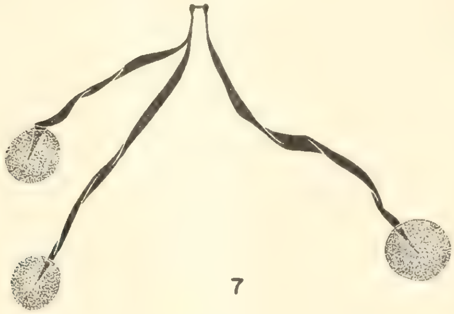

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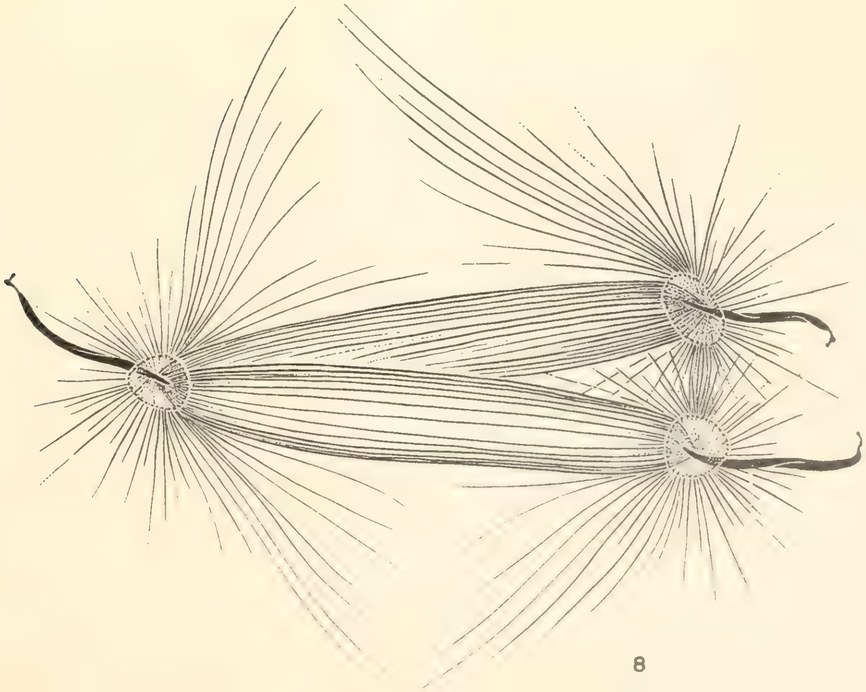

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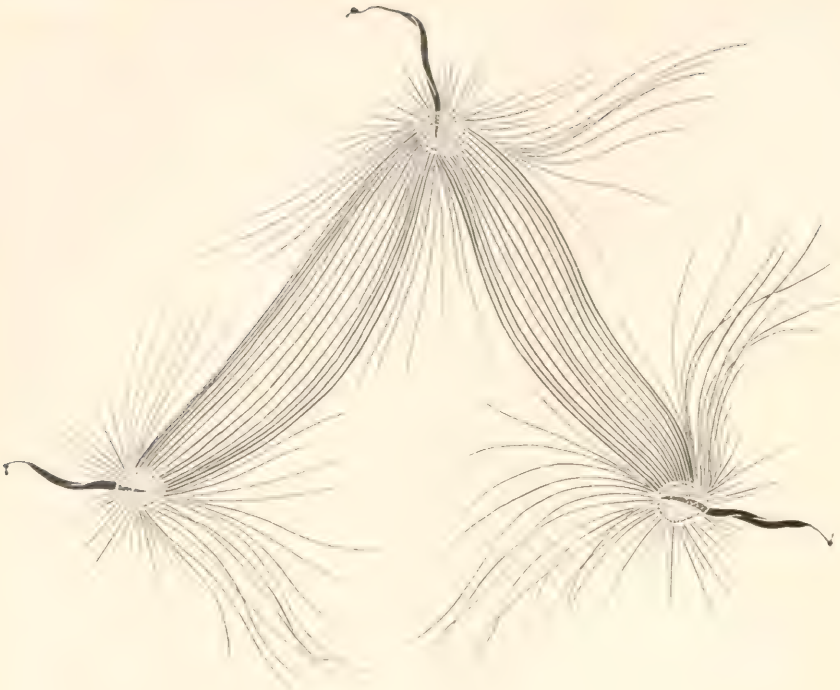

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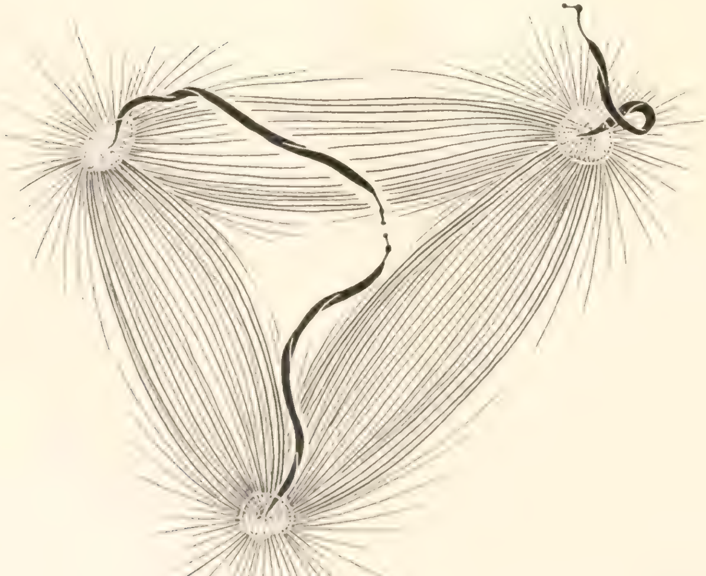

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PLATE 3

FIG. 9. Three centrioles in another position and the achromatic figure produced by them. × 1600.

FIG. 10. Still another position of three centrioles and the achromatic figure produced by them. × 1600.

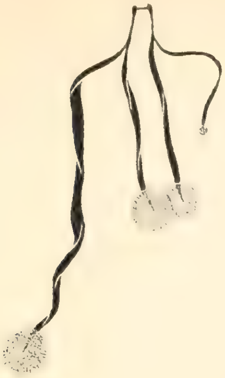
Plate 4

Fig. 11. Four interphase centrioles, one mature and three immature. × 1400.

Fig. 12. Five centrioles and their achromatic figure. × 1400.

Fig. 13. Four centrioles and their achromatic figure. Note the criss-crossing of the astral rays forming the central spindle. × 1400.

Fig. 14. Two centrioles so far apart that their astral rays could not meet to form the central spindle portion of the achromatic figure. × 600.



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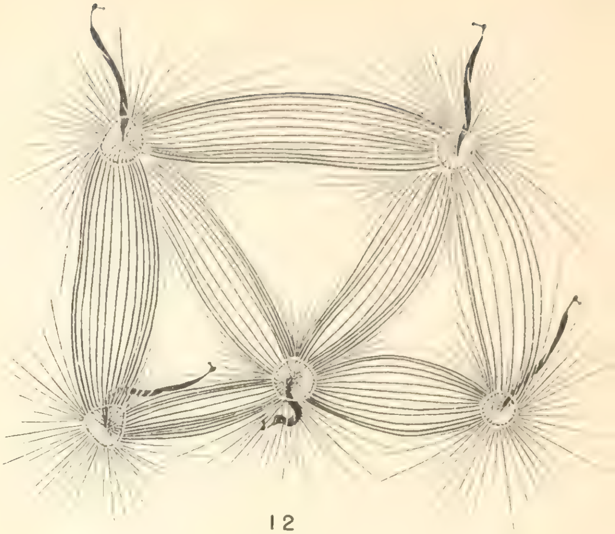

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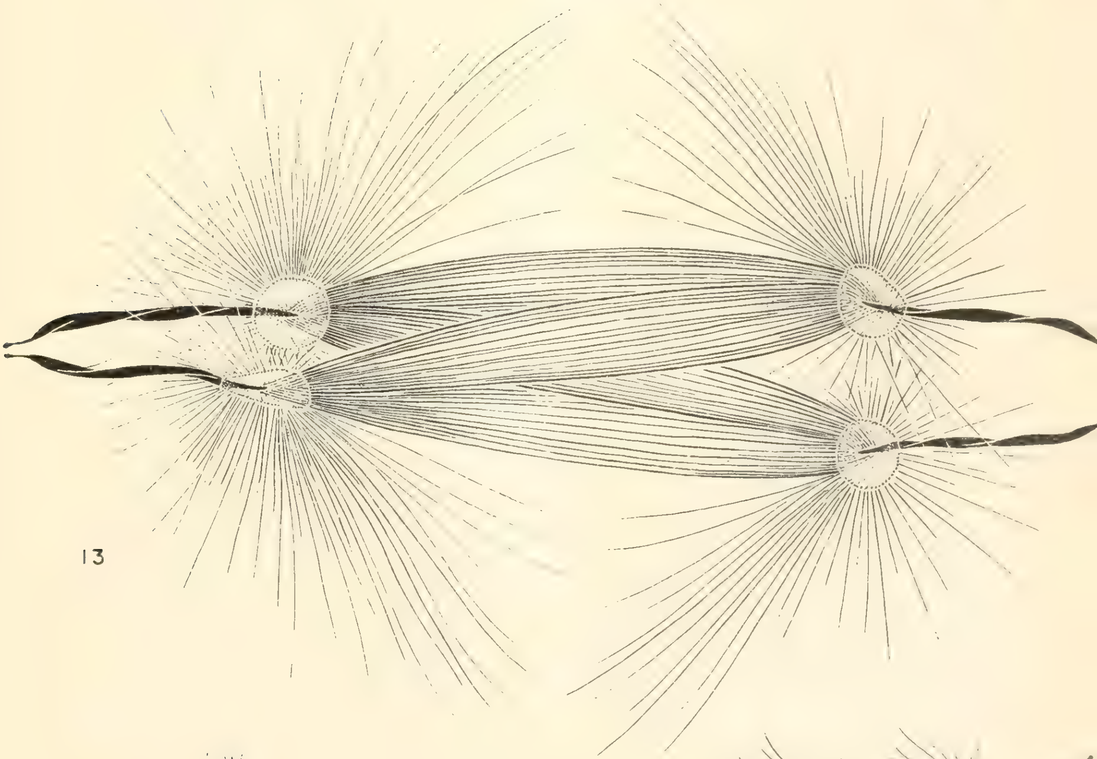

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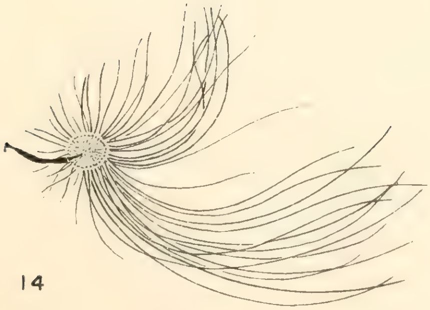

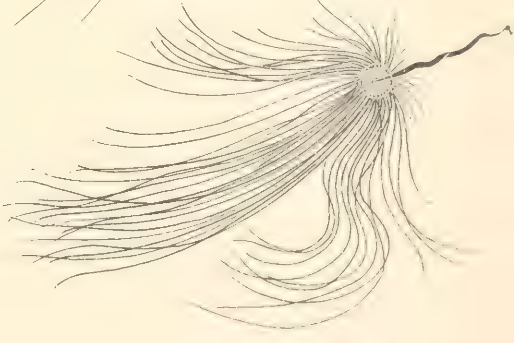

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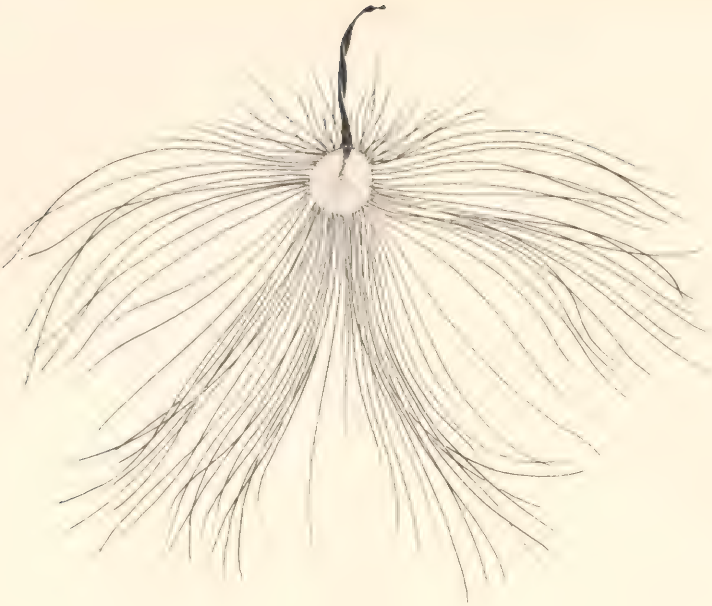

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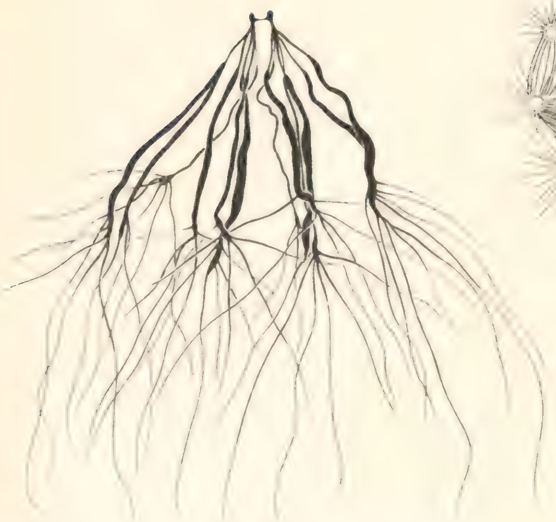

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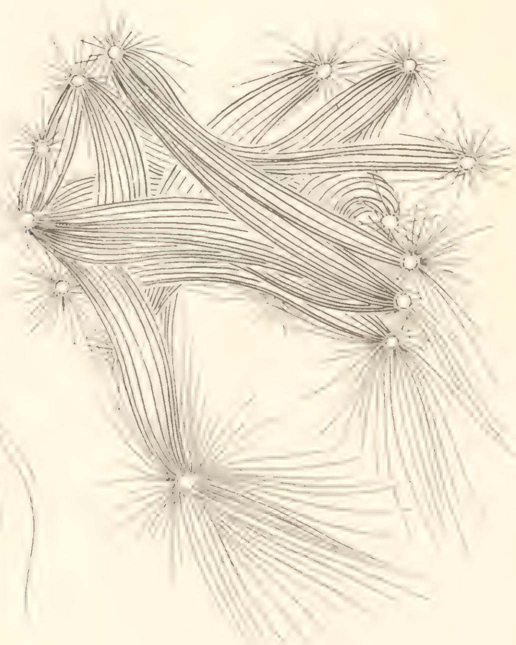

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PLATE 5

FIG. 15. The type of achromatic figure produced when only one centriole is present. Note the absence of a central spindle. × 1400.

FIG. 16. Fifteen centrioles are functioning in the production of an achromatic figure. In order to avoid confusion, the centrioles are omitted; but the position of their distal ends is indicated by the position of the centrosomes. × 1400.

FIG. 17. Interphase centrioles with probable secondary posterior extensions. × 1400.

THE ADSORPTION OF BACTERIA BY MARINE BOTTOM[1]

SELMAN A. WAKSMAN AND UNTO VARTIOVAARA

(From the Woods Hole Oceanographic Institution and New Jersey Agricultural Experiment Station)

In various recent studies (1, 4, 5) on the adsorption of bacteria in soil, the impression is left that the bacteria exert, in that state, only little effect upon the cycle of life in the soil. In his book on Agricultural Microbiology, Chudiakov (2) states emphatically that the problem of the condition of the bacteria in the soil hampered considerably a better understanding of soil microbiological processes. The existence of a surface relationship between the soil and microörganisms is true not only of bacteria but also of Protozoa, as shown by Cutler (3).

Different species of bacteria were found to behave differently in regard to their adsorption by the soil particles; their mobility and ability to form zoöglea seemed to be of special significance in this connection. The chemical activities of the bacteria, as measured by the evolution of CO_2, were considerably modified by the adsorption process (4). The finer soil constituents, namely the clay and silt fractions, were found to have a much greater adsorptive effect than the sand. Different soil types were found to adsorb bacteria to a different degree (5).

Rubentschik (8) made a detailed study of the phenomenon of bacterial adsorption in salt basins; the bacteria isolated from the mud were found to show a higher degree of adsorption than the bacteria found in the water. The conclusion was reached that the bacterial benthos consist of easily adsorbable species whereas the bacterial plankton contains organisms possessing a low degree of adsorption by the bottom sediments. A bacterial exchange took place in the mud, the adsorption of some species being accompanied by the desorption of others from the mud. The relative concentration of the bacteria, their nature and the type of bottom material exerted an influence upon the adsorption process. The activities of different species of bacteria were variously modified in the adsorbed state: the metabolism of some was lowered and that of others was increased.

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[1] Contribution No. 161 of the Woods Hole Oceanographic Institution and Journal Series Paper of the New Jersey Agricultural Experiment Station, Department of Soil Microbiology.

According to Peele (6), the adsorption of bacteria is probably due to the attraction of unlike electric charges. The nature of the base in the soil complex influences considerably the process of adsorption, the monovalent cations showing the least adsorption.

In most of these studies, pure cultures of bacteria were used and the results obtained interpreted in terms of processes carried out by a complex population of microörganisms inhabiting the soil or the sea bottom. Usually a very short period of contact was allowed between the soil and the bacterial culture grown upon an artificial medium; no attempt was made to determine what happened after the culture had adjusted itself to the environment.

These studies have an important bearing upon the influence of the sea bottom upon the bacterial activities in the water. A number of questions may arise in this connection; 1. Is the specific occurrence of certain bacteria in the bottom due to their adsorption by the bottom material? 2. Is the relatively low number of bacteria in the sea water, as compared with that in the bottom, due to their removal from the water by the adsorption process? 3. Is this relationship responsible for the difference in the rate of bacterial processes taking place in shallow seas over a sand bottom such as Georges Bank, compared with the corresponding processes over a mud bottom, such as the Gulf of Maine (7, 11)?

These and other questions deserve fundamental treatment. No attempt will be made to give a definite answer in the following experiments, which must be considered as preliminary in nature.

Experimental

In order to eliminate the interfering effect of the bacterial population normally found in fresh bottom material, the first experiments were carried out with marine mud and sand which had been kept in a dry state for 5 years and in which the bacteria had been reduced to very low numbers.

In the first experiment, the adsorption of bacteria from a mixed population and from a pure culture of a marine bacterium by two different types of dry marine mud and sand was studied. A mixed culture was obtained by allowing fresh sea water to remain, in a glass container, in the laboratory, for a period of 48 hours. This resulted in an increase in the numbers of bacteria from a few hundred to 303,000 per 1 cc., as determined by the plate method. As a pure culture, a marine agar liquefying bacterium (No. 11) was used. This organism was selected because of the ease of recognizing the colonies produced on the plate. It had been isolated from sea water and kept in culture for a period of 2 years. The organism was grown in a medium poor in nutrients (1 gram peptone, 1 gram glucose and 0.5 gram K_2HPO_4 in one liter of sea water), for 24 or 48 hours. The culture was then diluted ten times with sea water sterilized by heating 30 minutes at 80° C. The two muds, No. 1329 and No. 1331, contained 2.46 and 1.58 per cent organic carbon and 0.28 and 0.16 per cent nitrogen, respectively (9). The sand contained 0.58 per cent carbon and 0.05 per cent nitrogen. Ten-gram portions of mud or sand were placed in 250-cc. flasks containing either 100 cc. cultured sea water or 100 cc. of the diluted 24-hour culture of the bacterium. The flasks were shaken by hand for 10 minutes, allowed to stand 10 minutes, and 1-cc. portions of the supernatant liquid plated out using a sea water agar medium (No. 1). The flasks were kept in the laboratory, with occasional shaking, and water plated out again after 2 and 21 hours.

TABLE I

Adsorption of bacteria from mixed and pure cultures by dry marine mud and sand

Nature of culture	Bacteria in 1 cc. water, thousands			
	Start	*10 minutes*	*2 hours*	*21 hours*
Cultured water				
Control	303	—	—	—
Plus mud 1329	—	28	13	3,700
Plus mud 1331	—	25	75	530
Plus sand	—	180	115	32,000
Bacterium No. 11				
Control	1,600	—	—	—
Plus mud 1329	—	9,620	6	5
Plus mud 1331	—	13,000	21	365
Plus sand	—	22,000	40,000	40,000

The results presented in Table I show that the pure culture of the bacterium was readily adsorbed by the marine mud. The sand, however, had no adsorptive effect; there was, in fact, a marked increase in bacterial numbers due to the separation of the bacterial masses or clumps into individual cells as a result of shaking with the sand. In the case of the crude cultures (i.e. the bacterial population in the cultured sea water), the immediate adsorption of the bacteria was followed by a rapid increase in numbers. The latter was due to the rapid multiplication of certain bacterial forms at the expense of the organic matter in the mud and sand.

This experiment was repeated and an attempt made to measure not only the multiplication of the bacteria, but also their activities, as influenced by the presence of bottom material. This could best be done by determining the oxygen absorption, as a result of bacterial multiplication (10). Two-gram portions of the two muds and 5-gram portions of the sand were placed in oxygen bottles of about 220 cc. capacity. The bottles were filled with 3-day-old cultured sea water. They were closed and incubated at room temperature, under water and in the dark. At different intervals, some of the bottles were removed and analyzed for bacteria, by plating out 1 cc. of the supernatant water, and for oxygen by the Winkler method.

The results reported in Table II show that there was comparatively little adsorption of bacteria from the mixed bacterial population in cultured sea water, by either marine mud or sand. On the contrary, there was a marked rise in bacterial numbers, reaching a peak in 17 to 42 hours, followed by a rapid decline. Active oxygen absorption took place in the bottles accompanying bacterial multiplication; this was due to the oxidation of the organic matter in the bottom material by the bacteria.

TABLE II

Influence of marine bottom material on the growth and metabolic activity of a bacterial sea water population

Nature of culture	Bacteria in 1 cc. water, thousands					
	Start	*3 hours*	*17 hours*	*42 hours*	*4 days*	*8 days*
Sea water control	0.5	0.9	1.2	1.0	7.3	3.1
Plus mud 1329	1.8	1.9	1,175.0	850.0	133.0	12.2
Plus mud 1331	1.9	1.7	1,240.0	1,435.0	205.0	3.3
Plus sand	2.2	3.9	1,255.0	615.0	65.0	3.3
	Oxygen consumed, cc. per liter					
	Start	*3 hours*	*17 hours*	*42 hours*	*4 days*	*8 days*
Sea water control	0.0	0.03	0.07	0.23	0.38	0.64
Plus mud 1329	0.0	0.25	0.73	1.42	2.53	3.48
Plus mud 1331	0.0	0.13	0.62	1.29	2.47	3.31
Plus sand	0.0	0.19	0.88	1.57	2.18	3.24

A study was now made of the adsorption of bacteria from mixed and pure cultures by the use of relatively large amounts of bottom material. In this experiment, the mud and sand were placed in large test tubes and sterilized by heat at 120° C. for 1 hour. Two volumes of cultured water, 2 days old, or two volumes of a diluted 2-day-old culture of No. 11 were added to the sterile tubes. These were now shaken for 1 minute and allowed to settle for 10 minutes. The supernatant liquid was plated out immediately. The tubes were then allowed to remain in the laboratory for 24 hours; some were shaken occasionally and some were left undisturbed. The water was plated out after 24 hours. The results (Table III) show that there was again a rapid adsorption of the bacteria from the pure culture by the mud but not by the sand. In the mixed culture there was at first a certain amount of adsorption of the bacteria; this was followed by a very rapid increase in numbers. The large amount of mud used offered a good source of energy, especially after heating, for many of the bacteria in the water.

A comparative study was now made of the adsorption of bacteria by fresh marine mud obtained from Buzzards Bay. Varying amounts of the fresh mud, containing 75 per cent moisture, were placed in 250-cc. Erlenmeyer flasks. Some flasks received 100 cc. portions of 2-day-old sea water, while other flasks received 100 cc. of a diluted 2-day-old culture of No. 11. All flasks were shaken for 1 minute, allowed to settle for 10 minutes and the supernatant liquid plated. This was repeated after 1, 2, 4 and 10 days. Some of the flasks were kept undisturbed, while others were shaken at various intervals. The plates were incubated at room temperature for 48 hours and counted.

TABLE III

Influence of shaking upon the adsorption of mixed and pure cultures of bacteria by sterile marine bottom material

Nature of culture	Bacteria in 1 cc. of surface water, thousands		
	Shaken		Unshaken
	Start	*24 hours*	*24 hours*
Cultured water control	4.6	153.0	170.0
Plus mud 1329	0.3	3,600.0	39,000.0
Plus mud 1331	0.1	12,000.0	22,000.0
Plus sand	100.0	10,000.0	7,000.0
Bacterium No. 11 * control	33,000.0	214,000.0	130,000.0
Plus mud 1329	3,300.0	4,400.0	31,000.0
Plus mud 1331	30.0	1.0	1.0
Plus sand	39,000.0	320,000.0	220,000.0
Bacterium No. 11 † control	13,000.0	21,000.0	21,000.0
Plus mud 1329	0.7	1.0	1.0
Plus mud 1331	30.0	1.0	1.0
Plus sand	18,000.0	18,000.0	25,000.0

* Dilution of culture with sterile sea water in ratio of 1 : 10.

† Dilution of culture in ratio of 1 : 30.

The results (Table IV) show that fresh marine mud exerted at first a reducing effect upon the numbers of bacteria in the mixed population.

After 24 hours, however, there were more bacteria in the water above the mud than in the control water sample. It is interesting to note that the larger the amount of mud used, the higher was the number of bacteria, especially in the shaken mud, in spite of the fact that the immediate reduction in numbers was greater with an increase in the amount of mud. This was due to the fact that the bacteria found in the mud a source of nutrients and began to multiply rapidly. This confirms the results obtained previously by the use of dry mud and sand.

In the case of the pure culture, there was active adsorption of the bacteria by the fresh marine mud, but here also the bacteria began to multiply after 24–48 hours. These were not, however, the typical colonies of the agar liquefying organism added to the mud, but various colonies of bacteria commonly found in the marine bottom.

Table IV

Adsorption of bacteria by fresh marine mud

Nature of culture	Fresh mud *	Shaking	Numbers of bacteria in 1 cc. water, thousands				
	grams per 100 cc.		*Start*	*24 hours*	*48 hours*	*4 days*	*10 days*
Cultured water	0	0	53	10	9	9	<1
Cultured water	2	0	44	17	4	3	<1
Cultured water	10	0	42	10	32	23	6
Cultured water	50	0	24	32	50	13	2
Cultured water	0	+	45	16	11	21	<1
Cultured water	2	+	30	13	27	29	17
Cultured water	10	+	33	16	23	36	62
Cultured water	50	+	23	13	21	79	80
No. 11	0	0	12,700	140,000	240,000	236,000	310,000
No. 11	2	0	11,900	23,000	3,000	2,500	58
No. 11	10	0	13,900	1,000	2,000	3,600	182
No. 11	50	0	17,900	5,900	6,000	1,800	41
No. 11	0	+	14,700	160,000	332,000	310,000	360,000
No. 11	2	+	16,600	67,000	23,000	6,700	160
No. 11	10	+	15,400	13,000	6,000	1,100	92
No. 11	50	+	15,000	4,400	1,000	68	104

* Moisture content of mud 75 per cent.

On the basis of these results, one can hardly conclude that the adsorptive action of the mud bottom upon bacteria in the water is detrimental in any manner to their activities. Even though mud adsorbs, removes, or inactivates a large number of bacterial cells such as may be obtained by growing a pure culture of a bacterium upon an artificial medium, there is still no proof that mud as such is in any manner injurious to bacterial processes in general, either those taking place in the water or in the mud itself. The evidence presented here seems to point merely to the fact that bottom mud exerts a certain controlling effect upon the nature of the bacterial population in the sea water. This is, of course, true of the bacteria found in the benthos or in the water immediately above it, but may not hold at all for the plankton bacteria found at great distances from the bottom.

The results of these experiments further emphasize the fact that the phenomena of adsorption obtained by the use of pure cultures of bacteria grown upon artificial media are difficult to interpret in terms of natural marine processes, since the conditions controlling the relations of the mud bottom to the natural mixed population of bacteria in sea water are much more complex.

Summary

1. Marine mud exerted an adsorptive effect upon the bacteria in sea water. This effect was particularly evident when certain pure cultures of bacteria were added to the mud to give a heavy inoculation. Sand bottom had very little adsorptive action upon either mixed or pure cultures of bacteria.

2. In the case of the mixed bacterial population in the water, the adsorption of the bacteria by the mud was soon followed by a rapid increase of bacterial numbers; this took place at the expense of the organic matter in the bottom material.

3. These laboratory experiments showed that certain types of marine bottom material exerted a controlling effect on the numbers of bacteria, which were in intimate contact with them. There was no indication, however, of a permanent paralysis of bacterial growth or metabolism.

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PRIMARY SEXUAL PHASES IN THE OVIPAROUS OYSTER (OSTREA VIRGINICA)

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In a recent paper on environment and sex in this species (Coe, 1936*a*), data were presented to show that the sex ratio at the first breeding season is correlated with age and size at sexual maturity. The proportion of individuals first functioning as females was found to be greatest in those localities and during those seasons most favorable for rapid growth. The mean size of the young females is larger than that of males of the same age, as Needler (1932*a*) had previously reported.

During the past year additional evidence has been obtained from other localities along the coast from Long Island Sound to the Gulf of Mexico. This evidence seems to justify certain more or less definite conclusions in regard to the factors which regulate the sexual changes which individuals of this species are known to undergo during their lifetime. Through the courtesy of Dr. Victor L. Loosanoff the writer has had the opportunity of examining additional extensive collections from Milford, Connecticut, while Professor T. C. Nelson has kindly furnished an abundant supply of young oysters of definitely known age from the culture frames of the New Jersey laboratory, on the shore of Delaware Bay, near Cape May. Dr. H. F. Prytherch, of the Bureau of Fisheries, has supplied additional material from Beaufort, North Carolina, and Dr. A. E. Hopkins has sent additional samples from the Gulf Coast laboratory at Apalachicola, Florida. The data concerning the sexual conditions found in this new material are included for comparison with those from other localities in Table I.

Examination of large numbers of fully adult oysters shows that there is usually an approximately equal number of individuals functioning at the time as males and females, or with an excess of females among the older members. The young oysters, on the other hand, always show a large excess of males at their first spawning season. This initial tendency toward maleness is the result of the more rapid growth and multiplication of the male than of the female constituents in the primary reproductive gland in a majority of the young individuals of this alternosexual species (Coe, 1932*a*, 1934).

From Cape Cod to the Chesapeake Bay, most of the young oysters may be expected to spawn at the age of one year. North of Cape Cod the first spawning is stated to occur more often at the end of the second year, as is the case with some individuals in certain years south of Cape Cod. On the coast of North Carolina and in the Gulf of Mexico, well nourished individuals of the early set spawn toward the end of their first summer, when only three to four months of age, while those of the later set do not become mature until the following spring.

TABLE I

Observed sex ratios during first breeding season at different localities and in different years

Locality	Date	No. undetermined	No. M.	No. H.	No. F.	Total	Ratio F. to 100 M.
New Haven Harbor	7-11-32	17*	389	4	13	423	3.34
" " "	7-27-33	1*	129	0	7	137	5.42
" " "	8-2-35	105*	521	4	40	670	7.66
" " "	7-28-36	3*	327	2	41	373	12.54
Milford (Bed)	8-1-34	2*	80	1	6	89	7.50
" "	7-20-35	2*	312	2	75	391	24.04
" "	7-3-36	77*	780	10	44	911	5.64
" "	8-13-36	7	410	4	58	479	14.15
" "	8-25-36	176†	92	0	21	289	22.83
Milford (Gulf)	7-7-37	0	116	3	25	144	21.55
Milford (Harbor)	7-7-37	10*	306	7	57	380	18.60
" "	7-28-37	6†	116	2	29	153	25.00
Great South Bay, L. I.	7-31-32	21*	197	0	7	225	3.55
West Sayville (float)	1932	0	154	4	48	206	31.17
Delaware Bay	7-7-37	0	520	13	218	751	41.92
Beaufort, N. C.	1933	0	43	0	21	64	48.84
" "	8-10 to 10-20-36	10	84	2	34	130	40.48
" "	10-20-37	73*	151	3	56	283	37.09
Apalachicola, Fla.	1936, 1937	81*	127	0	9	217	7.09
	Total	591	4854	61	809	6315	16.66

* Sexually immature.

† Spawned out.

The ratio of the two sexes at their first breeding season varies greatly from year to year and at different localities, as shown in Table I. It also varies at different parts of the season, not only because the males tend to become mature somewhat earlier than the females but also in consequence of an actual change in the sexuality of some of the individuals because a small percentage of the young oysters function as males in the early part of the season and as females immediately thereafter (Coe, 1936*a*). A few others are hermaphroditic and capable of self-fertilization.

This is well illustrated by three collections of yearling oysters from Milford, Connecticut, kindly supplied by Dr. Victor L. Loosanoff, of the Bureau of Fisheries, in 1936. A sample of 911 yearlings examined at the beginning of the breeding season contained less than 6 recognizable females for each hundred males, in addition to 10 hermaphrodites and 77 small individuals that were sexually immature. At the middle of the season, however, the ratio of females had increased to 14 and near the end of the season to nearly 23 (Table II). In 1937 there was likewise an increase in the proportion of females in the later of two collections from the same locality (Table II). Since the females are often somewhat later in maturing than are the males, they frequently spawn later. Hence, if it may be assumed that the immature individuals found early in the season contained a large proportion of potential females and that the majority of those spawned out when the last examination was made had previously functioned as males, the differences in the observed sex ratios of the several collections would have an adequate explanation.

TABLE II

Change of sex ratio during first breeding season

Locality	Date	No. undetermined	No. M.	No. H.	No. F.	Total	Ratio F. to 100 M.
Milford (Bed)	7-3-36	77*	780	10	44	911	5.64
" "	8-13-36	7*	410	4	58	479	14.15
" "	8-25-36	176†	92	0	21	289	22.83
Milford (Harbor)	7-7-37	10*	306	7	57	380	18.60
" "	7-28-37	6†	116	2	29	153	25.00

* Sexually immature.
† Spawned out.

An examination of some 4,500 young oysters from Long Island Sound during six successive years has shown a ratio of from $3\frac{1}{3}$ to 25 females for each hundred males, depending upon the local conditions and the season (Table I). The average size of the females exceeds that of the males and as a general rule those seasons and situations most favorable for rapid growth have shown the largest percentage of females.

Different culture beds in near-by situations may differ considerably in the ratio of the sexes in the yearling population. Those in shallow, warmer situations may spawn some weeks in advance of those less favorably situated. The first set may have a more favorable as well as a longer growing season than later ones, with a correspondingly larger proportion of females. Morphological sex differentiation may occur in the autumn in the one case or it may be delayed until winter in the later sets.

Since there is usually found an approximate equality in the ratio of the sexes or an excess of females after the second year, it is obvious that at least 25 to 45 per cent of the young males in these localities must change to females. It is not yet definitely known whether any of the yearling females change to males but there is conclusive experimental evidence that both males and females sometimes change their sex in the interval between two breeding seasons even at the age of four or more years (Needler, 1932*a*, Galtsoff, 1937). The stimuli which inaugurate these sexual transformations have not been determined, but it is suspected that both inherent tendencies and nutritional conditions may be involved.

The cellular mechanism involved in this change of sexual phase has been shown (Coe, 1932*a*, 1934) to depend upon the presence of gonia of both sexual characteristics or undifferentiated gonia through one sexual phase and their retention as residual cells after spawning. In some species of oysters and other mollusks an alternating rhythm of male and female phases is of regular occurrence (Coe, 1936*c*).

Summarizing the total collections from each area, as shown in Table I, it may be noted that the collections from New Haven Harbor for the four recent years in which suitable material could be obtained comprised 1,066 males and 101 females, a ratio of 9.6 females for each 100 males. From Milford, only nine miles distant, a total of 2,212 males and 315 females were contained in the eight collections during four summers. The sex ratio would there average 14.2 females for each 100 males, indicating a generally more favorable condition for the young oysters than in New Haven Harbor. This conclusion is supported by the fact that the average size of individuals of the same age was somewhat larger at Milford than at the latter locality.

From a natural bed in Great South Bay, Long Island, an exceptionally low ratio of females was found in 1932, while from culture floats not far distant, the ratio was nearly nine times as great (Table I) corresponding with a similar increase in the rate of growth.

Farther to the southward the ratio of females is still greater under favorable conditions, averaging more than forty for each one hundred males both on the culture frames in Delaware Bay and at Beaufort, North Carolina (Table I).

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Correlation between Sexuality and Size at First Breeding Season

The more recent studies are quite in harmony with the evidence presented by Needler (1932*a*), Burkenroad (1931*a*), Coe (1932*c*), Roughley (1933) and others that the mean size of individuals functioning as females at the first breeding season is considerably greater than that of males of the same age. In an unselected sample consisting of 751 yearlings from the culture frames at the laboratory on the shore of Delaware Bay, 358 had a shell length of 50 mm. or more on July 7, 1937, while 393 were smaller. Examination of the gonads showed a ratio of 63.4 females for each 100 males in the group of larger individuals as compared with 26.6 females for each 100 males among the smaller ones. This correlation between sexuality and size for these 751 young oysters is shown graphically in Fig. 1. It will be noted that none of the females was less than 25 mm. in length and

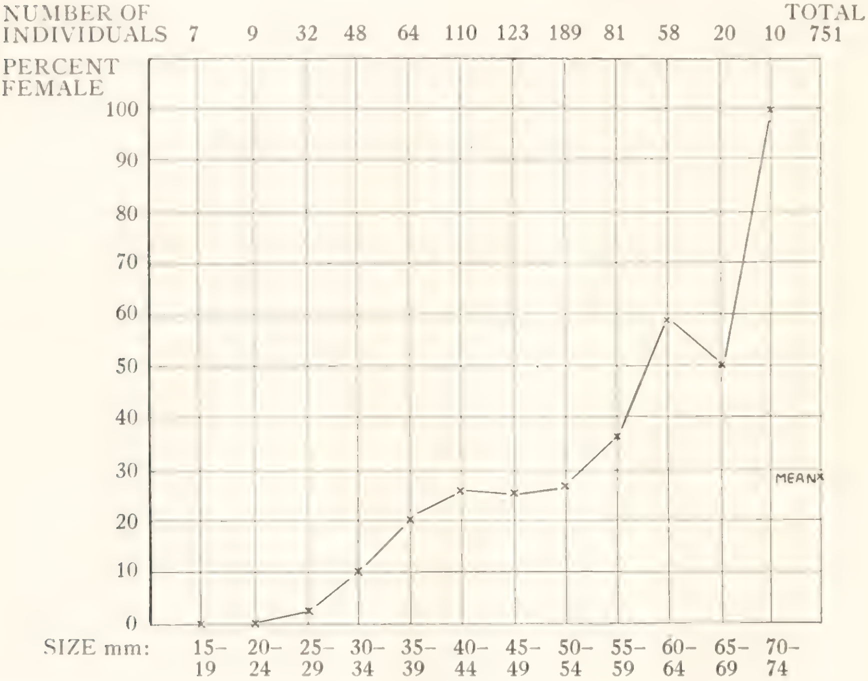

FIG. 1. Graph showing correlation between sexuality and size at first breeding season, based on an unselected sample of 751 yearlings from the culture frames at the New Jersey laboratory on the Shore of Delaware Bay near Cape May. The size of the class is shown at the bottom of the graph and the number of individuals in each class at the top. The mean percentage of females in the entire sample is also indicated.

none of the males more than 69 mm. The largest number of individuals, however, were between 40 and 55 mm. in length and in these groups there was little difference in the percentage of females.

Direct Female Development

The primary gonad may be either distinctly bisexual in appearance or it may become differentiated very early into either of the two sexual phases (Coe, 1932*a*). In the latter case there is a direct transformation of the primary undifferentiated gonia into the functional sexual cells. The female phase is then attained without indication of protandry.

In Long Island Sound the sexual phase of the first breeding season is sometimes distinguishable as early as October or November in exceptionally well nourished young at the age of only three or four months after setting. More frequently, however, morphological sex differentiation is delayed until mid-winter or later.

At Beaufort, North Carolina, direct female development appears to be the general rule, with a much higher ratio of young females than in more northern localities (Table I). Samples of well nourished individuals from Delaware Bay, known to have set July 9, 1936, likewise indicated direct development, when examined at the end of December. No evidence of incipient protandry was found at that time, although in many cases the gonads were sexually differentiated.

Indirect Female Development

It has been shown previously (Coe, 1932*a*), that the primary gonads of some young individuals develop directly into ovaries or spermaries, as the case may be, while those of other individuals of the same age are distinctly bisexual, or, to use a less ambiguous term, ambisexual.

The latter are characterized by a cortical layer of ovogonia and young ovocytes, with spermatogenic cells at one side or in the lumen. In many cases these ovocytes later degenerate or they may remain inactive during the primary functional male phase, to become activated and functional toward the end of that phase or some time thereafter. In this way the sexual phase may change from male to female either during the first breeding season or, much more frequently, during the following autumn.

A subsequent change from female to male phase evidently results from the later activation of some of the descendents of the primary undifferentiated gonia into spermatogonia. The propagation of such undifferentiated gonia may be continued year after year or even through a long lifetime, since some residual gonia always remain after spawning.

It was also previously reported (Coe, 1932*a*) that the developing gonads exhibit all intergradations between those of the so-called true males in which no indications of ovogonia or ovocytes can be detected and those which develop directly into ovaries. In a few individuals both types of cells in the ambisexual gonad multiply harmoniously, leading to the functionally hermaphroditic condition at the breeding season.

In northern localities some of the spermatogonia in the young ambisexual type of gonad may undergo transformations during the autumn comparable with those taking place in normal spermatogenesis (Coe, 1932*a*). These cells may later degenerate, followed by the activation of ovogonia, thus leading to the functional female phase at the first breeding season during the following summer. Such incipient protandry seems to be much less frequent than the direct development in which the initial dominant phase, either male or female, is retained during the winter and the following breeding season.

Sexual Conditions in Southern Localities

From North Carolina southward the initial sexual phase in the earliest set of the year becomes functional toward the end of the same season, when the young oyster is only three or four months of age, as Burkenroad (1931*a*) reported for the Louisiana oyster. Young of the later sets are similar to those of more northern localities in their sexual conditions, although protandry may be less frequent (Table I). Thus at Beaufort, North Carolina, and at Apalachicola, Florida, on the Gulf of Mexico, there may be two generations of this species in one year, individuals of the early set spawning some three to four months after setting while those of later broods do not become ripe until the following spring.

Cultures in both these localities are usually contaminated with more or less numerous individuals of the larviparous species, *O. equestris*, which matures at a still younger age and has a breeding season covering most of the year in warm situations. It is difficult to distinguish the two species externally when young, but the relations of the epibranchial chamber differ and the gonads are very different, since *O. equestris* has a sequence of overlapping male and female sexual phases and the spermatogenic cells are larger than in *O. virginica* and arranged in dense clusters.

A small collection of the over-wintered young at Beaufort in 1933, kindly supplied by Dr. H. F. Prytherch, showed a ratio of nearly 49 females to 100 males, while the first set of the season in 1936, stated to have occurred about June 1, showed a female ratio of about 40 when examined in August, September and October. The largest individuals had a shell length of 23 mm. in August, 40 mm. in September, and 70 mm. in October. In August there were only a few males with ripe sperm and toward the end of October most individuals of the early set were spawned out.

Collections of the early set taken September 20, 1937, about fifteen weeks after setting, were from 20 to 50 mm. in length and showed a female ratio of 37.09 (Table I). Those which exceeded 35 mm. in length had a female ratio of about 61 as compared with 16 for those of smaller size.

An unselected sample containing 88 individuals which were one year old, originating from the autumnal set during the last two weeks in September, 1936, on the other hand, showed a ratio of 75 females to 100 functional males. These were doubtless in their second spawning season, having previously participated in the spring spawning during June, 1937, when seven to eight months of age. The ratio of females was thus about twice as great as was found in the young at the first spawning period. This would indicate that the proportion of young males changing to the female phase for their second spawning period must have been about 22 per cent greater than the number of individuals, if any, which experienced a sex change in the opposite direction during the same time.

Small collections from Apalachicola, Florida, obtained through the courtesy of Mr. R. O. Smith, were examined three to five months after setting in 1936. These, combined with an additional collection kindly supplied by Dr. A. E. Hopkins from the same locality in 1937, showed a female ratio of 7.09 (Table I), corresponding closely to the ratios from northern localities under unfavorable conditions.

Discussion

The foregoing evidence concerning the sexual conditions in the Virginia oyster indicates that the sex-differentiating mechanism must be in a very labile condition since it responds so generally to environmental influences.

Because of the different sex ratios under different environmental conditions, it seems probable that the responses noted may depend upon the interaction of several associated factors, both genotypic and phenotypic. Among these it is evident that all these populations have at least the three following categories of hereditary and environmental influences.

(1). There is an inherent tendency toward protandry, as is the case with so many other mollusks, both pelecypods and gastropods, including the larviparous oysters (Orton, 1927; Coe, 1932*b*), *Teredo* (Coe, 1936*d*), *Venus* (Loosanoff, 1937), *Patella* (Orton, 1928), and *Crepidula* (Coe, 1936*b*, 1938), to mention only a few of the numerous examples. This tendency may or may not be realized, since direct female development so frequently occurs under favorable conditions.

(2). There appears to be an inherent tendency toward rhythmical change in the sexual phase during successive breeding periods, as in the larviparous oysters, *Teredo*, and certain gastropods. The fully adult Japanese oyster (*Ostrea gigas*) was proved by Amemiya (1929) to be subject to this change in sexuality in the interval between two breeding seasons. In the Virginia oyster a corresponding condition has been found by Needler (1932*a*, 1932*b*), by Burkenroad (1937) and by Galtsoff (1937). The proportion of the adult population in which this change occurred varied greatly in the different experiments, the discrepancies being due presumably both to varying environmental conditions and to comparatively small samples. The most extensive of these experiments is that by Galtsoff, who found that among 202 fully adult oysters of which the functional sexuality was reliably determined during the breeding season of 1936, only 167 retained the same sexual phase in 1937. Of the remainder, 14 had died in the meantime, while 9 or 14 per cent of the females had become functional males, and 11, or 8.9 per cent of the males were found to be in the female phase in 1937. The twenty individuals which experienced this change of sexuality during the interval between the two breeding seasons represented only 10.7 per cent of the total number of survivors.

It is evident, then, that sexual stability may predominate over any tendency toward rhythmical change much more definitely than is the case with the larviparous species. There is also considerable evidence which indicates that the sexual rhythm in the latter is associated with a metabolic rhythm (Coe, 1934). But the nutritive conditions are certainly modified by environmental influences. Change of sexual phase in *Crepidula*, like metamorphosis in other animals, has been shown recently (Coe, 1938) to be subject to environmental control, and this is presumably true to some extent in the oyster.

Immediately after spawning, the residual gonia in both the sexual phases show considerable variation. Some individuals which have spawned as males show residual small ovocytes in addition to apparently undifferentiated gonia. Others show only spermatogonia and undifferentiated gonia, while females may retain few or many or no young ovocytes after the cytolysis of the larger ovocytes. In both sexual phases cytolysis may proceed until only undifferentiated gonia remain and the individual has become essentially neutral.

Evidently relatively few fully adult individuals have gonads that are so evenly balanced that slight changes in the internal environment may activate either type of the residual gonia and thus determine the sexual phase of the following season. In the majority of both sexes, the same sexual phase is retained (Amemiya, 1929; Needler, 1932*a*; Galtsoff, 1937; Burkenroad, 1937).

(3). Since the primary gonads in the young oyster before morphological sex differentiation show great variability in the proportion of cells characteristic of the two sexual phase, it seems probable that these are to some extent at least dependent upon different heritable modifying factors (Coe, 1932*a*, 1934, 1936*a*). Functional males, hermaphrodites and females may then be expected under uniform conditions, the proportion of each depending upon the combination of factors present in each individual. But in the population of young oysters in any locality preceding the time or primary sexual differentiation, in the autumn, winter or summer, according to the locality, even a generally uniform environment will be highly variable in so far as each individual is concerned, since there are differences in age, in situation relative to other individuals and the like (Orton, 1936).

The larger average size of the females at the time of sexual differentiation and later and the higher ratio of females in those years and in those localities or situations most favorable for rapid growth may indicate either (*a*) differences in hereditary factors or (*b*) a direct response sexually to nutritive and other conditions, or (*c*) both. The evidence to date seems to imply that both these influences are operative.

The sexual conditions in other species of oviparous oysters are similar to those here discussed for the Virginia oyster. In the Indian oyster (*O. cucullata*) Awati and Rai (1931) found a corresponding tendency toward protandry, but with an excess of females in the adult population, which averaged 41 per cent males, 3 per cent hermaphrodites and 56 per cent females. Those which harbored the commensal crab *Pinnotheres*, however, showed 82 per cent males, 7 per cent hermaphrodites and only 10 per cent females, indicating a masculinizing influence on the part of the commensal, either nutritive or otherwise. Roughley (1933) likewise found a very strong tendency toward protandry in the Australian oyster (*O. commercialis*), but the adult population consisted of 54 to 88 per cent females, averaging about 2.7 times as many females as males. He considered that nutritive conditions were not influential in determining the sexual phase, since populations under poor conditions for growth showed about the same ratio of females as those having better nutritive conditions. The Portuguese oyster (*O. angulata*) is another species in which the females are more numerous than the males in the adult population, Orton and Awati (1926) reporting ratios of 60 to 88 per cent females. Indeed, in relatively few species of fully adult pelecypods are the males as numerous as the females (Pelseneer, 1935).

Amemiya's studies on change of sex in the Japanese oyster (1929) include later experiments (1935) in which a portion of the gills was removed from adult oysters immediately after spawning. The operation was performed on four groups of oysters, with an equal number of controls. The following year there was a slightly larger ratio of males in each one of the operated groups than in the controls. It was concluded that the proportion of individuals assuming the male sexual phase was influenced by the decreased facility of the operated individuals in obtaining food.

All these considerations emphasize the complexity of genotypic and phenotypic factors which may be influential in controlling the sexual phases of the Virginia oyster. The evidence here presented indicates that the populations along the coast from New England to the Gulf of Mexico consist of several local races living under widely different environmental conditions and differing in some respects in their inheritance. In the more northern localities a certain proportion of the young oysters function as females during their first breeding season at the age of about twelve months either by direct female development or indirectly after passing through an abortive or, less frequently, a brief functional male phase. In southern localities two generations, of which the second comprises a large percentage of females, may occur within the same twelve months period.

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THE DELAYED OCCURRENCE AND TOTAL OMISSION OF ENDOMIXIS IN SELECTED LINES OF PARAMECIUM AURELIA

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Experimental control of the process of endomixis in *Paramecium aurelia* has been only partly achieved. Jollos (1916), Sonneborn (1937*b*) and others have developed methods of inducing endomixis, but no method of avoiding it completely is known. Temporary suppression of endomixis was probably attained by Jollos (1916); but the method employed volumes of culture medium so great as to make it impossible to ascertain with reliability whether endomixis was occurring or not. The present paper sets forth a method that avoids this difficulty and provides not only some lines of descent with greatly extended interendomictic intervals, but also others that never go into endomixis.

The method is based on the observation (Sonneborn, 1937*a*) that the interendomictic interval varies greatly in sister lines cultivated under the same conditions. An attempt was therefore made to select the lines with the longer intervals. This was done by discarding lines as they went into endomixis and replacing them by new lines begun with surplus individuals from sister lines that had not yet gone into endomixis. When this is done with a group of 12 to 24 daily isolation lines cultivated under the conditions employed by Sonneborn (1936), the following phenomena occur. At first, none of the lines go into endomixis; then, as the "normal" time for endomixis approaches, more and more of the lines go into endomixis and have to be replaced by their sister lines that have not yet gone into endomixis. During a period of several weeks, this high frequency of endomixis continues and necessitates equally frequent eliminations and replacements; but during the following few weeks the frequency of endomixis greatly decreases and thereafter occurs but rarely. Selection has thus been effective in seeking out lines in which endomixis completely fails to occur. However, such lines cannot be continued indefinitely; after four or five months they die.

This method has been successfully applied in this laboratory many times and is now a routine technique. As illustrations of the results obtainable with it, two typical histories will be given in the following

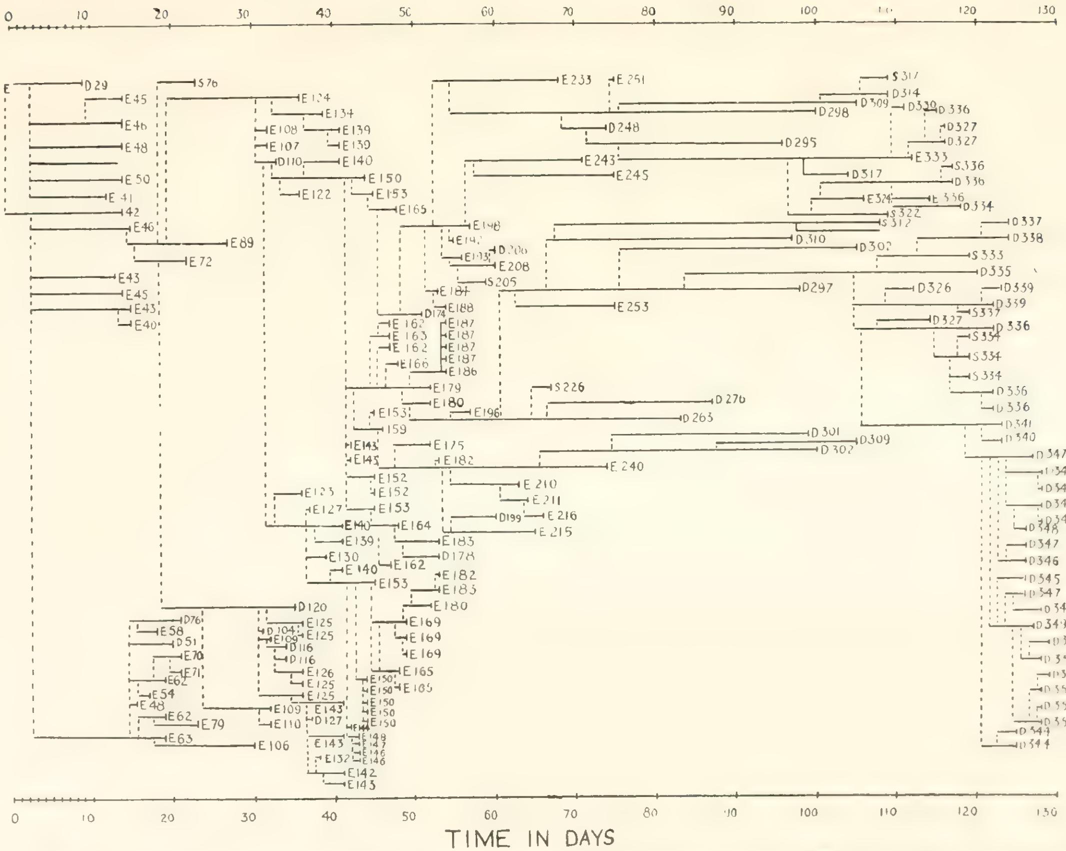


FIG. 1. Selection of non-endomictic lines in race *R*, *Paramecium aurelia*. The solid horizontal lines represent daily isolation lines of descent. The broken vertical lines connect sub-cultures with their source cultures. *E* symbolizes endomixis; *D*, died; and *S*, killed and stained after two days without fission. The numbers following *E*, *D*, and *S* give the number of fissions without endomixis in the direct line of descent from the original progenitor in endomixis on the first day to the final individual which terminated each sub-culture.

two sections: one of these involves animals of the Johns Hopkins race *R*, reported upon in recent papers by the author and associates; the other involves animals of Woodruff's long-lived race, here designated *W*. In the third section, the relation of these results to the variability of the interendomictic interval is set forth.

Race R

Figure 1 gives the results of applying the method of selection to a group of typical lines of race *R*. The entire group was derived from one individual in endomixis April 14, 1935. The original 12 lines descended from this individual went into endomixis between the 14th and 20th days (after 41 to 63 fissions) following the initial endomixis.

TABLE I

The frequency of endomixis and of death in relation to the time since the last preceding endomixis in races R and W of *Paramecium aurelia*

Race	Days since preceding endomixis	Number of lines in endomixis per 100 line-days	Number of deaths per 100 line-days
R	1–13	0	0.8
	14–55	23.3	2.6
	56–76	6.4	2.4
	77–122	0.6	5.9
	123–130	0	35.8
W	1–30	0	2.0
	31–76	3.8	0.8
	77–130	0.5	4.8
	131–163	0	13.4

Each of these lines was replaced at the time of endomixis by a new line started with a surplus individual from one of the sister lines that had not yet gone into endomixis. By repeating this process of replacement in all the descendant lines whenever endomixis occurred or might have occurred, some of the lines lived for as much as 130 days (350 fissions) without endomixis.

As shown in Table I, the life of this group of lines fell into five periods differing markedly in the frequency of endomixis and death. The first period, extending to the 13th day, was characterized by the complete absence of endomixis. In the second period, from the 14th to 55th days, many lines went into endomixis and had to be replaced. The third period, from the 56th to 76th day, was marked by a great reduction of the number of lines going into endomixis. In the fourth period, from the 77th to 122nd day, only three lines went into endomixis, but the number that died or stopped multiplying increased. Finally, in the fifth period, from the 123rd to 130th day, no line went into endomixis, but the death rate was so high that the group completely died out. In Table I, lines which stopped multiplying and were found, on staining, not to be in endomixis, are included among those which died. Experience showed that this was their usual fate.

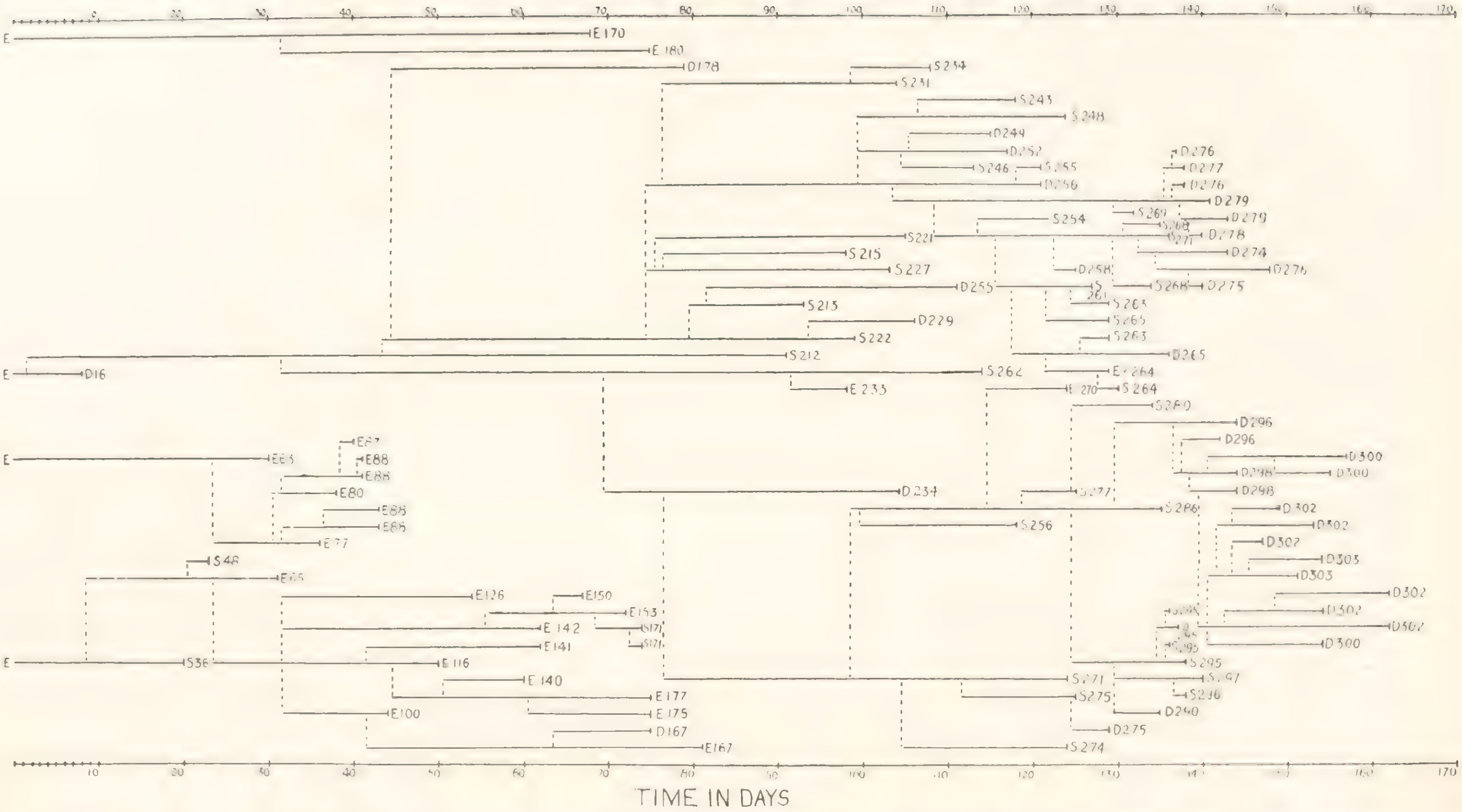
The main feature of the preceding account is that endomixis did not occur in certain lines of descent carried through 350 fissions during 130 days. This was demonstrated by cytological studies. Each line of descent was stained on every day that fission occurred; and if fission failed to occur in any line for two successive days the line was stained and so brought to an end. Since every line that showed any nuclear condition even remotely suggesting endomixis was discarded, it is certain that no endomixis was overlooked and that the lines living through till the end of the experiment had not experienced endomixis during the entire time.

Race *W*

The method of obtaining non-endomictic lines of descent was also applied successfully to race *W*, though here the detailed results differ from those obtained with race *R*. A typical set of results on race *W* is illustrated by Fig. 2. The group there represented began with four individuals in endomixis on February 10, 1935. As shown in Table I, the life of this group is divisible into four distinct periods. In the first period, extending to the 30th day, there was no endomixis and few deaths occurred; in the second period, from the 31st to the 76th day, the frequency of endomixis was at its highest and the death rate was still low; in the third period, from the 77th to 130th day, there were few endomixes (two certainly, and possibly four), but the death rate showed a marked increase; in the fourth period, from the 131st to 163rd day, there were no further endomixes and the death rate reached its peak, resulting in the extermination of the group.

As in race *R*, the lines of race *W* which were carried to the end of the experiment showed no endomixis during the entire period of observation. Their life without endomixis extended for 163 days and included 302 successive fissions. This long period is $5\frac{1}{2}$ times as long as those reported to be typical for this race by Woodruff and Erdmann (1914) and it includes about seven times as many fissions.

Comparison of the data for the two races given in Table I shows that the group of race *W* lines had a longer initial period without endomixis (30 days as compared with 13), and that it at no time attained so high a rate of endomixis or death as did the group of race *R* lines. Further, the maximum period without endomixis was longer in race *W* than in race *R*, but included fewer fissions.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TIME IN DAYS

FIG. 2. Selection of non-endomictic lines in race W, *Paramecium aurelia*. The solid horizontal lines represent daily isolation lines of descent. The broken vertical lines connect sub-cultures with their source cultures. *E* symbolizes endomixis; *D*, died; and *S*, killed and stained after two days without fission. The numbers following *E*, *D*, and *S* give the number of fissions without endomixis in the direct line of descent from the original progenitors in endomixis on the first day to the final individual which terminated each sub-culture.

Three other races (*H*, *E*, and *S*) have been subjected to the same method of selection with similar results. It seems reasonable to suppose that what has been accomplished in these five races could probably be done with most or all races of *P. aurelia* under similar cultural conditions: by selection, lines can be isolated that do without endomixis throughout their lives—a period 5 to 7 times as long as the ordinary interval between successive endomixes.

Variability of the Interendomictic Interval

In the absence of selection, the extent of the period during which no nuclear reorganization occurs was shown by Sonneborn (1937*a*) to vary greatly. The preceding sections show that still greater variability appears when the selection technique is applied. This greater variability must be due to the existence of relatively rare lines which, without any special experimental treatment, have extremely long interendomictic intervals or even fail completely to undergo endomixis. Since such lines are very rare, they would ordinarily not be found in work where the investigator is able to observe only a small sample of a race. The method of selection serves simply to seek out these lines and multiply them. Without resorting to any change of cultural conditions, it provides a means of obtaining both lines that long omit endomixis and lines that omit it entirely.

SUMMARY

If daily isolation lines of *P. aurelia* are regularly discarded as soon as they go into endomixis, and if these are then replaced by sister lines that have not yet gone into endomixis, it is possible to maintain for long periods lines which have not been in endomixis since the start of this procedure. With this method there were obtained lines of race *R* which omitted endomixis for as long as 130 days and 350 fissions, and lines of race *W* which omitted endomixis for 163 days and 303 fissions. At the end of these long periods, all lines died.

In the culture of groups of lines selected in this way, there is an initial period during which endomixis does not occur; this is followed by a period in which endomixis occurs in many of the lines (which have to be discarded); during the remaining history, endomixis occurs rarely and eventually not at all, but the death rate rises and results in the final extinction of the group. The details as to the duration of these periods and their characteristic death and endomixis rates differ in different races. The method of selection emphasizes the enormous variability of the interendomictic interval. The extremely long intervals are very rare, and the selection method is simply a device for finding these rare lines and multiplying them.

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THE DISTRIBUTION OF AMMONIA IN THE WATERS OF THE GULF OF MAINE

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In the decomposition of organic residues in the sea ammonia is apparently the first simple inorganic compound of nitrogen to be set free. Since it does not commonly occur in large quantities, it is evidently oxidized rapidly to nitrate and nitrite. As an ephemeral intermediate in the cycle of regeneration of nitrate, it is of interest in indicating the places in which the decomposition of nitrogenous material is taking place.

The present paper presents the results of a survey of the occurrence of ammonia in the waters of the Gulf of Maine based on observations made during cruises of the "Atlantis" in September, 1933 and May and June, 1934. The only previous measurements of ammonia in these waters of which we are aware are those made by Seiwell (1931) in Penobscot and Frenchman's Bays and those recorded by Rakestraw (1936) in a study of the occurrence of nitrite in the sea. Our results are correlated with the studies of nitrite made by Rakestraw of the phosphorus cycle by Redfield, Smith and Ketchum (1937), and with observations on the distribution of plankton, based on collections made during these cruises.

METHODS

Ammonia was determined by the Teorell titration of the vacuum distillate as described by Krogh (1934). The apparatus could be used practically on shipboard, using alcohol lamps burning pure alcohol for heating and a Cenco Hyvac pump as a source of low pressure. Ammonia-free water was prepared ashore. It was found necessary to steam out the apparatus before the start of each series of analyses and to make blank analyses between each pair of unknowns. To be reliable, all determinations must be made in duplicate, since unforeseen contamination frequently occurred.

The sea water samples were taken in Nansen reversing bottles, from which they were immediately transferred, with double rinsing, to glass-stoppered bottles. These were stored in the refrigerator and analyzed as quickly as possible. Experiments recorded in Table I showed that sea water stored for about eight hours underwent no appreciable change in ammonia content. With longer periods of storage there is a progressive decrease in the ammonia content, which is not prevented by the addition of 0.1 per cent mercuric chloride (Keys, Christensen and Krogh, 1935). The reason for this loss is unknown.

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[1] Contribution No. 159.

RESULTS

The positions of the stations at which reasonably complete and satisfactory observations on the distribution of ammonia were obtained in September, 1933 and May, 1934 are shown in Figs. 1 and 2. Observations were also made at six stations in the Gulf between June 25 and June 30; the results obtained at that time did not differ from those of a month earlier except that on the whole smaller amounts of ammonia were present. In presenting the results it is convenient to separate the observations made in deep water where the stability of the water column preserves its heterogeneity from those obtained in shallow water and in the shallower channels across the banks where a high degree of turbulence prevails.

TABLE I

Stability of ammonia in stored sea water samples *

First analysis		Second analysis		Third analysis	
Hours after collection	NH_3 N. mg./M^3	Hours after collection	NH_3 N. mg./M^3	Hours after collection	NH_3 N. mg./M^3
1	40	7	41	290	10
2.5	27	8.5	19	290	8
3.0	35	7	35	290	15
2.5	28	8	30	290	2
4	20	—	—	90	13
4	62	17	54	90	45
6	42	—	—	90	25
6	44	18	24	—	—
6	57	18	46	90	37
5	49	17	28	90	23
4	46	17	32	—	—
5	42	18	30	90	20
†1.5	36	290	8		
†2	25	290	4		
†2.5	21	290	15		
†8.5	31	290	16		

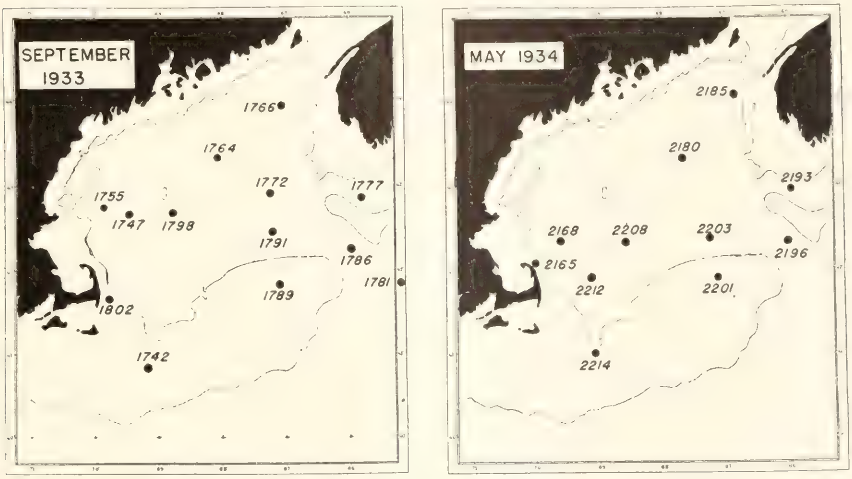
* All values are averages from duplicate analyses.

† $HgCl_2$ added to sample at time of collection.

Observations in Deep Water

Figure 3 represents the concentrations of ammonia at various deep stations in September and in May. The stations are arranged as far as possible in the order of the positions through which the water is thought to circulate on penetrating the Gulf. Stations occupied in May are placed under stations occupied in comparable positions in September.

In September, Station 1781, 45 miles southeast of the Eastern Channel, showed the presence of some 10 mg. N per cubic meter in the upper 60 meters, diminishing to zero at 100 meters, below which level no ammonia was detected down to the depth of 2,000 meters. The ammonia in the water in the Eastern Channel was similar in distribution, there being none observable below 125 meters. Within the Gulf, on the other hand, the concentration of ammonia was distributed rather uniformly throughout the water column. The concentrations of ammonia occurring at any station increased towards the western basin of the Gulf, where amounts exceeding 50 mg. N per cubic meter were found.



Figs. 1 and 2. Position of stations at which distribution of ammonia was determined in September, 1933 and May, 1934 respectively.

In May the distribution of ammonia was very different. Amounts in excess of 10 mg. N per cubic meter were rarely observed in the upper 20 meters and at depths greater than 100 meters. Between these depths, at 30 to 60 meters, maximal concentrations occurred ranging in value from 10 mg. N per cubic meter in the Eastern Channel to 45 mg. N per cubic meter in the southern part of the western basin. In the following month similar conditions were observed save that the maximal concentrations did not exceed 25 mg. N per cubic meter.

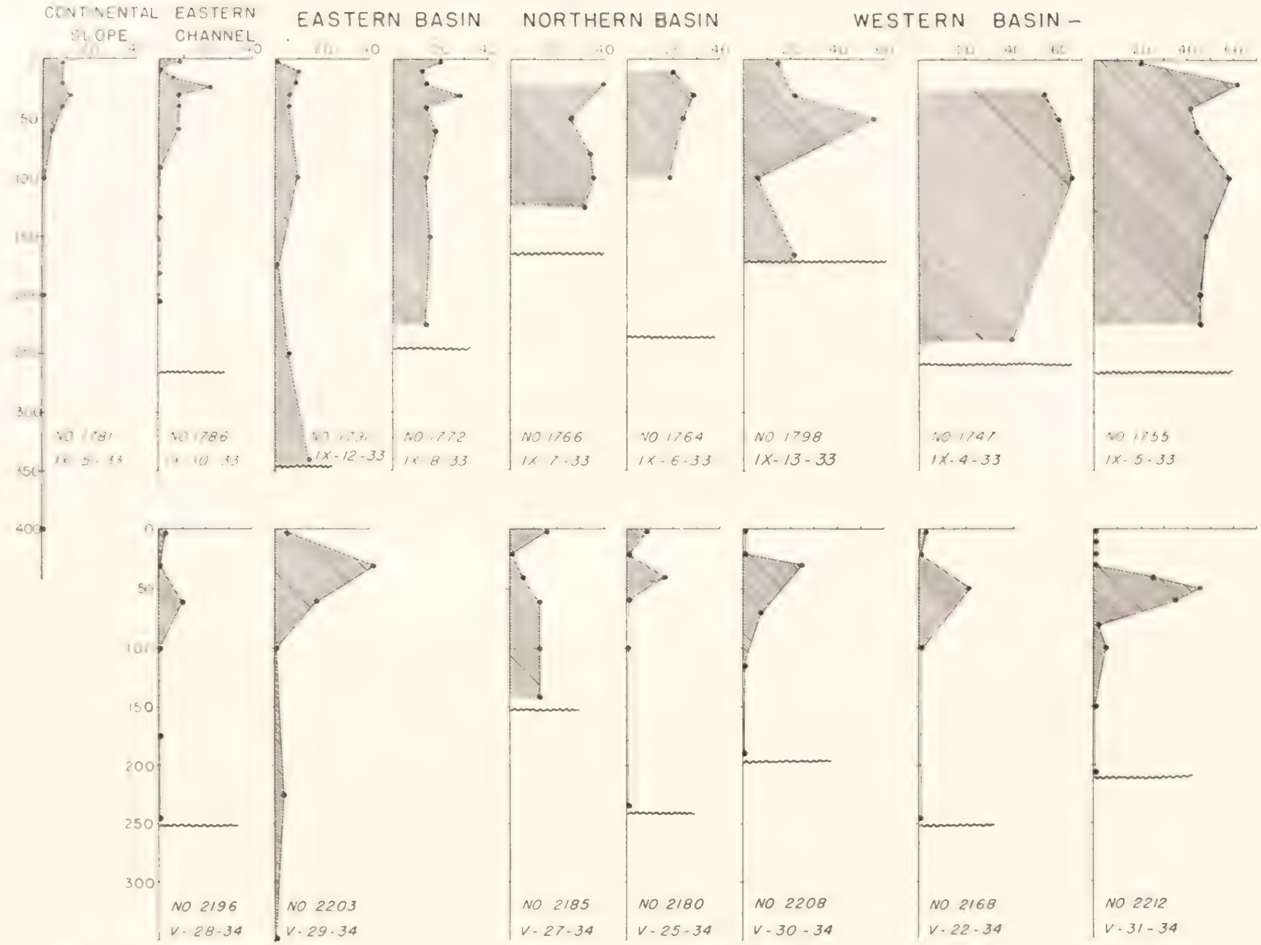


FIG. 3. Vertical distribution of ammonia in the deeper waters of the Gulf of Maine. Upper row, in September, 1933; lower row, comparable positions in May, 1934. Depths measured in meters downward along the ordinate. Concentrations of ammonia expressed as milligrams ammonia nitrogen per cubic meter along the abscissa.

OBSERVATIONS IN SHALLOW WATER

Figure 4 shows the distribution of ammonia at shallow stations off Cape Cod and on Georges Bank and in the strong tide ways of the South and North Channels. No definite difference in the seasonal picture is evident. In the channels considerable concentrations of ammonia occurred at all depths, though in May a maximal concentration a short distance below the surface was present in the North Channel. In the shallow stations the concentration sometimes increased with depth, sometimes the reverse.

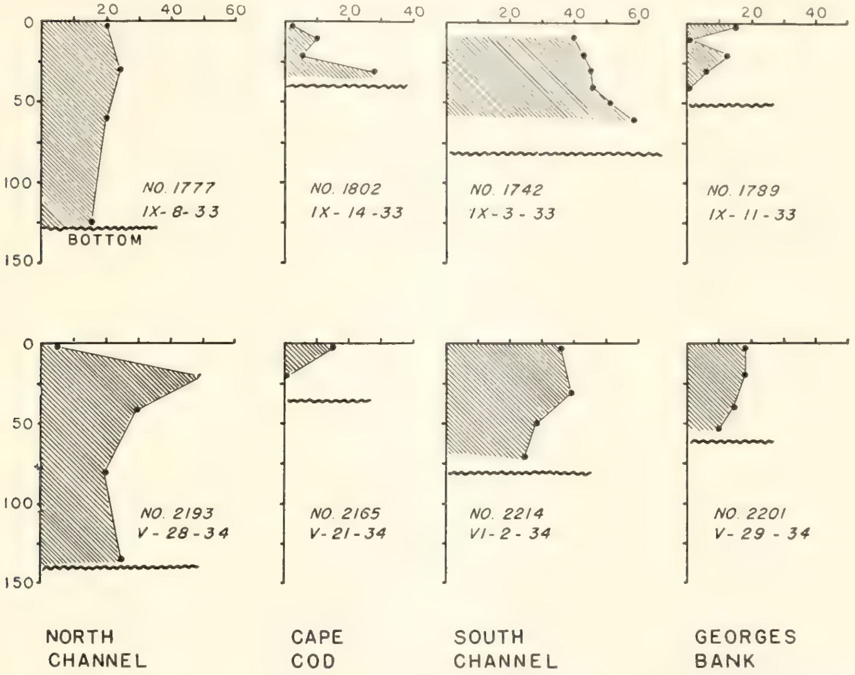


FIG. 4. Vertical distribution of ammonia in shallow waters of the Gulf of Maine. Upper row, in September, 1933; lower row, comparable positions in May, 1934. Concentrations of ammonia expressed as milligrams ammonia nitrogen per cubic meter along the abscissa.

DISCUSSION

The distribution of ammonia appeared very irregular when the observations were first made. The magnitude of the concentrations of ammonia observed agreed well with the general range of observations made by earlier workers in various regions (Robinson and Wirth, 1934). It appeared, however, that great differences might be observed in similar situations at any time and in the same situation at different times.

In several ways our observations support the conclusions drawn from similar studies of ammonia in the English Channel made by Cooper (1933). He considers the formation of ammonia to be a "surface" phenomenon with lesser activity at the bottom. In the spring the surface concentrations decrease from utilization by phytoplankton. Our May observations would appear to be adequately explained on these assumptions. He also observed a general increase in summer and autumn, as did we in September.

As additional evidence has accumulated concerning the biology and chemistry of the Gulf, a number of correlations appeared which seem to give the findings additional significance.

RELATION TO PHOSPHATE CYCLE

The most striking difference between the occurrence of ammonia in September and in May is its relative abundance in the deep water at the end of summer compared with its absence there in the spring. This is precisely the relation found for another intermediate product of decomposition—dissolved, organic phosphorus compounds. These were found by Redfield, Smith and Ketchum (1937) to disappear from the water of the western basin in mid-winter and gradually to accumulate during the summer, reaching a maximum observed concentration in November. Like the ammonia in September, the concentration was quite uniform at all depths. In the spring these phosphorus compounds appear in the subsurface layers, as does the ammonia, before they may be detected in considerable quantity in the depths. It will be of interest to observe whether the ammonia does not almost entirely disappear from the water in mid-winter, as does the dissolved organic phosphorus.

Like the organic phosphorus, ammonia appears to accumulate at all depths in late summer. This should not be thought of as the accumulation of an inert product, however, for von Brand, Rakestraw, and Renn (1937) have shown, at least in a laboratory experiment, that the ammonia of decomposing plankton is nitrified completely in 30 days. It is probable that the ammonia is formed by decomposition throughout the water column, for in September there are no well-marked gradients of ammonia concentration which are necessary for its transport by eddy conductivity. There is no evidence, except in a few of the shallow stations, of increasing concentrations near the bottom, such as Seiwell (1931) observed in shallow bays and as might occur if decomposition on the sea bottom was responsible for a considerable part of the regeneration of nitrogen.

RELATION TO NITRITE

If the view to which Rakestraw (1936) has recently lent support is correct—that nitrite arises in the water through the oxidation of ammonia—one would expect a close correlation in the distribution of these nitrogen compounds. This expectation is in part fulfilled. The distribution of ammonia in May closely parallels that of nitrite, as shown in Fig. 5. Both may be explained through decomposition taking place in the subsurface waters. Rakestraw explains the absence of nitrite from the water immediately below the surface as being due to the assimilation of this substance by plankton. Evidently ammonia is similarly consumed. It is interesting, though not surprising, that there is no evidence that soluble organic phosphorus compounds are assimilated in a similar way—since they were found in as high concentration in the surface as at any depth.

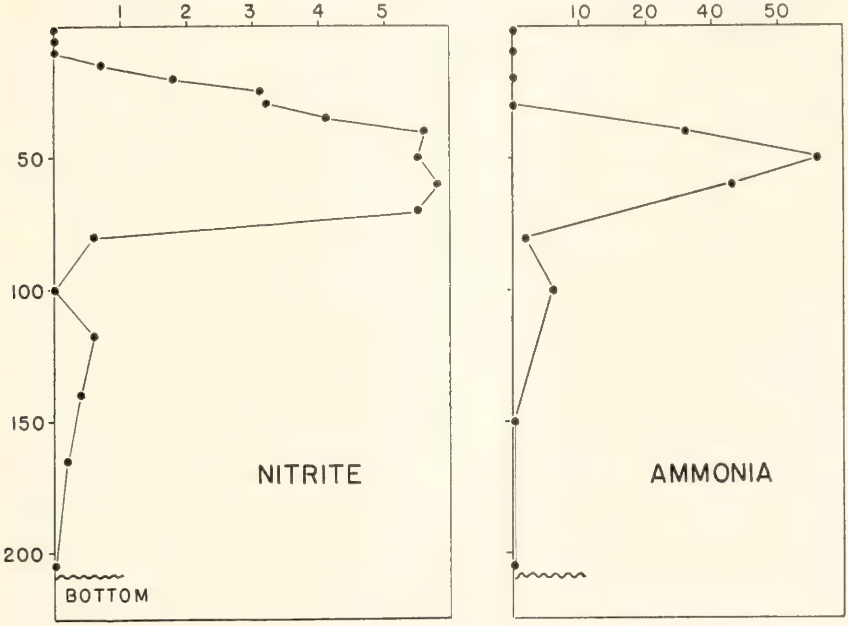


FIG. 5. Vertical distribution of nitrite and ammonia at Station 2212 May 31, 1934. Depths measured in meters downward along the ordinate. Concentrations measured in milligrams nitrogen present as nitrite or as ammonia per cubic meter.

Rakestraw remarks that the maximum for nitrite occurs between June and September. However, the nitrite does not appear to accumulate toward the end of summer to the extent to which ammonia and organic phosphorus compounds do. Nitrite is evidently a shorter-lived link in the chain of nitrogen transformations than is ammonia for even in May the ammonia nitrogen concentration exceeds that of nitrite nitrogen by ten-fold.

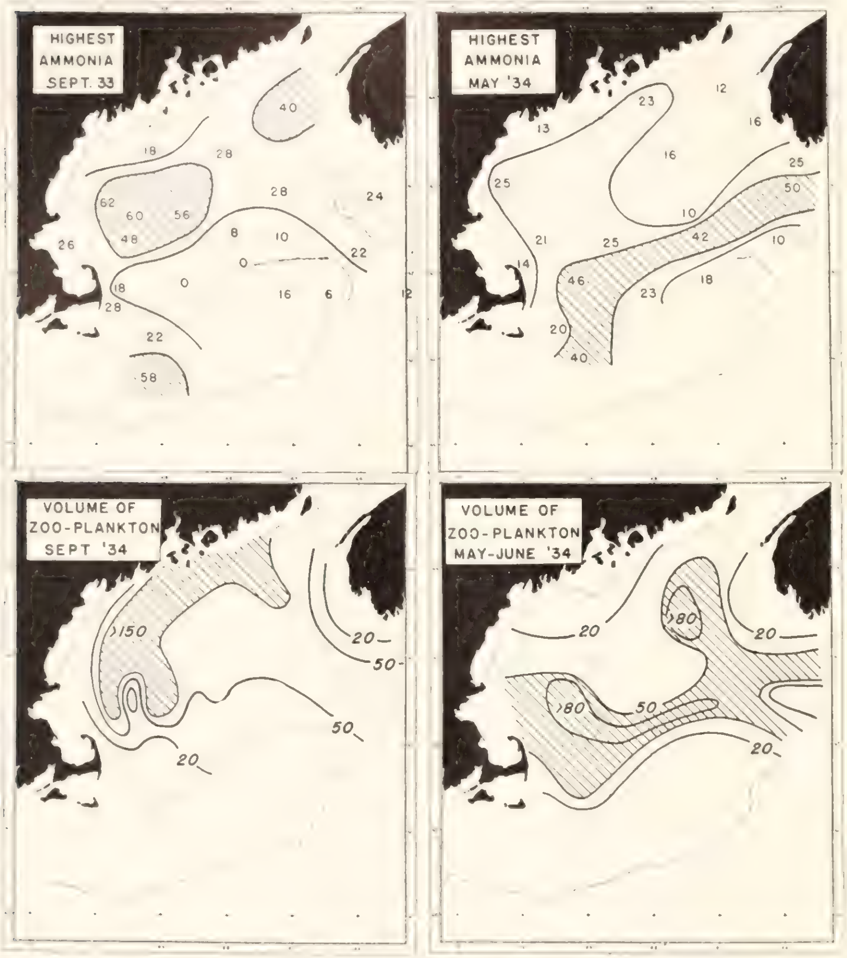

HIGHEST AMMONIA SEPT. 33
40
18
28
62
60
56
48
26
28
24
8
10
22
0
0
16
6
12
18
28
22
58
HIGHEST AMMONIA MAY '34
23
12
13
16
16
25
25
50
10
42
10
21
25
14
46
18
23
20
40
VOLUME OF ZOO-PLANKTON SEPT '34
>150
20
50
50
20
VOLUME OF ZOO-PLANKTON MAY-JUNE '34
20
>80
20
>80
50
20

Fig. 6. The highest concentration of ammonia in the upper 80 meters of water in various parts of the Gulf of Maine in September, 1933 and May, 1934 compared with the quantitative distribution of zoöplankton. Ammonia concentrations expressed in milligrams ammonia nitrogen per cubic meter. Zoöplankton volumes expressed as cubic centimeters of dry plankton per square meter of surface area caught in a vertical haul from near the bottom.

Relation to Plankton

The quantity of ammonia observed during both cruises varied greatly from station to station. In September the greater quantities were observed in the northern and western parts of the Gulf; in May in the southern and eastern regions. These facts did not assume significance until the plankton catches made at the time had been analyzed.

During each cruise a standard vertical zoöplankton haul was made at each station occupied (a number far exceeding those at which the ammonia was investigated). A 1.5-meter silk net was hauled from near the bottom to the surface. The total catch at each station was freed of excess moisture on a filter and its volume measured by displacement. The results of these measurements expressed as the volume of "dry" plankton per square meter surface have been plotted in Fig. 6 to show the general distribution of animal plankton. For comparison, the highest value of ammonia observed at each station in the upper 80 meters is also shown. While the correlation is not perfect, it is evident that the highest concentrations of ammonia at both periods of observation occur in regions in which the zoöplankton are densely distributed.

This correlation suggests that the animal plankton may be in some way responsible for the appearance of ammonia. While specific observations on plankton animals are lacking, ammonia appears to be the principal nitrogenous waste product of many invertebrate animals (Delauney, 1931). Harvey (1934) has pointed out that the phytoplankton community is continuously being grazed down by the zooplankton. These considerations point to the probability that zoöplankton, through their metabolic products and through their own decay, are an important intermediary in the liberation and distribution of ammonia in sea water.

SUMMARY

1. In the deeper basins of the Gulf of Maine in May ammonia occurred in minimal concentrations at the surface and at all depths below sixty meters; maximal concentrations varying up to 45 mg. N per cubic meter occurred in a definite stratum between 30 and 60 meters.

2. In September the concentration of ammonia was rather uniform at all depths and increased as the distance from the open sea increased, concentrations exceeding 50 mg. N per cubic meter occurring in the western basin.

3. In the tideways of the North and South Channels, ammonia is distributed uniformly with depth in both May and September.

4. In shallow waters its occurrence showed no regularity.

5. The occurrence of ammonia may be correlated in part with the distribution of organic phosphorus compounds, of nitrite, and of

zoöplankton, so as to support the view that its distribution marks the place and the intensity of organic decomposition.

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THE OXYGEN CONSUMPTION OF ARTIFICIALLY ACTIVATED AND FERTILIZED CHÆTOPTERUS EGGS

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INTRODUCTION

It is well known that unfertilized *Chætopterus* eggs, when they are treated with various physical or chemical agents, undergo a special type of development (F. R. Lillie's (1902, 1906) differentiation without cleavage). Lillie's original observations have been recently confirmed and completed by Pasteels (1934) and by myself (1937). These investigations show that if the unfertilized eggs are treated with 5 per cent isotonic KCl in sea water, they first complete their maturation; immediately after the expulsion of the second polar body, they start undergoing a series of monaster cycles. During each of the cycles, there is an increase in the number of the chromosomes and the appearance of lobulations simulating cleavage. But the furrows fade out quickly and the egg resumes its original shape. There is, therefore, a considerable increase in the size of the egg nucleus and in the number of the chromosomes during the first hours of differentiation without cleavage. Later on, the big single nucleus breaks down and, at the same time, occur processes comparable to gastrulation; in the best cases, unicellular ciliated eggs, resembling somewhat trochophores in their general shape, can be obtained.

It seemed of interest to measure the oxygen consumption of eggs undergoing differentiation without cleavage and to compare it with the respiration of unfertilized eggs: it was hoped that such an investigation would throw some light on the question whether energy is needed for cleavage and differentiation, a problem which has recently been investigated by Tyler (1933, 1936) on other eggs.

A beginning was made last year during a stay in the Zoölogical Laboratory in Naples and a report has been published recently (1937). The results, however, were far from satisfactory because a small percentage only of the eggs could be fertilized, although differentiation without cleavage was easily obtained. Lacking the necessary controls, we could only compare the data obtained for the activated eggs with the results published by Whitaker on the respiration of fertilized eggs in Woods Hole. Obviously, no definite conclusion can be drawn from such a comparison and it was therefore necessary to reinvestigate the problem.

MATERIAL AND METHODS

The experiments were carried out following the technical details pointed out by Whitaker (1933): one or two ripe females were allowed to shed after the tip of their parapodia had been cut with scissors. The eggs were then washed repeatedly (5 or 6 times) in sea water in order to remove the mucus and a suspension of a concentration varying from 1 : 25 to 1 : 40 was prepared by slightly packing the eggs in a graduated centrifuge-tube and adding the required amount of sea water. Aliquot parts of the suspension (2 cc.) were pipetted into Warburg manometer conical cups (ca. 10 cc. capacity). Fertilization with one drop of diluted sperm or activation with 0.1 cc. of isotonic KCl usually occurred just before attaching the vessels to the manometers. Readings were taken after a 15-minutes equilibration period. The water-bath was kept at 24.8° and the manometers were shaken at a speed of 50 round-trip oscillations per minute with an excursion of 4 cm. That these conditions may be considered as adequate is indicated by the fact that increase in the speed of shaking induced no increase in the oxygen consumption; furthermore, the respiration of unfertilized eggs usually kept constant during 6 or 7 hours and they showed at that time little, if any, cytolysis.

RESULTS

We know since Whitaker's experiments that there is a strong drop in the oxygen consumption of *Chætopterus* eggs at the time of fertilization. My previous experiments from Naples indicated that a similar drop occurs in KCl-activated eggs. In most of those experiments, activation was produced by tipping the KCl contained in the side-arm into the main part of the vessel after the respiration had already been measured during 1 or 2 hours. The results at Naples could be easily confirmed at Woods Hole: while the metabolism of the fertilized eggs dropped to 53 per cent of the initial level (average from 14 experiments in which the percentage of fertilized eggs exceeded 90 per cent), the oxygen uptake of the activated eggs (from the same females) fell to 51 per cent. There is therefore no doubt that, like the increase in respiration which follows fertilization in sea-urchin eggs, the drop observed in the present case is linked to the cytoplasmic changes resulting from activation and not to any special influence exerted by the spermatozoön on the metabolism.

The following graph represents the oxygen consumption of *Chætopterus* eggs during the 7 hours following activation and fertilization.

In this graph, the rate of oxygen consumption has been plotted against time, the initial respiration of the unfertilized eggs being considered as 100 per cent. The individual points on the curves represent average values obtained from 14 experiments.

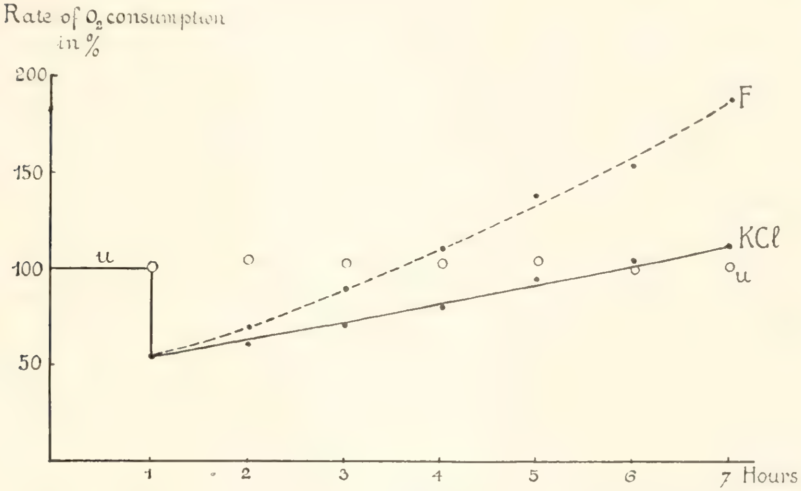

FIG. 1. Oxygen consumption of *Chætopterus* eggs during the seven hours following activation and fertilization. Ordinates: rate of oxygen consumption; abscissæ, hours. The curve *F* represents the oxygen consumption of fertilized eggs; the curve KCl represents that of eggs treated with KCl; and the circles, *u*, represent that for unfertilized eggs.

It is easy to see that, as stated before, the oxygen consumption of the unfertilized eggs (indicated by large circles) remains constant throughout the whole experimental period. It is evident too that respiration increases after the drop in oxygen consumption occurring after fertilization or activation, but obviously this increase is much stronger and faster in the case of the fertilized eggs than it is for the activated eggs: the fertilized eggs reach the initial level after 3½ hours, i.e. at an early blastula stage. The respiration rate increases steadily later on and is almost 80 per cent higher at the end of the experiment than the oxygen uptake of the unfertilized eggs. On the other hand, the activated eggs differentiating without cleavage reach the initial rate only after 6 hours instead of 3½. The fact that the same difference in the respiration of two sets of eggs was found regularly in all the experiments indicates beyond doubt that the observed fact is really significant.

It was possible that the difference might be linked to a depressive effect on the metabolism of the added KCl which was present during the whole experiment. In order to check this possibility, 4 series of control experiments were run in which the respiration of activated eggs was measured both in presence of KCl in excess as before and after repeated washings (10 times). In other manometers, the oxygen consumption of normal fertilized eggs was compared with the metabolism of similar eggs to which 5 per cent isotonic KCl was added 10 minutes after fertilization. All these control experiments showed that the presence or the absence of KCl in that concentration does not exert any significant effect on the curves. In both cases, the initial respiratory level was reached after 3½ hours in the case of the fertilized eggs and after 6 hours for the activated ones. It seems, however, possible that KCl slightly enhances the absolute O_2 consumption of the eggs. Obviously, the differences observed in the metabolic rate of eggs differentiating without cleavage and of fertilized eggs is somehow linked to their different type of development and not to a depressive effect of KCl on the metabolism.

It was also of some interest to see how the thymonucleic acid synthesis, as a chemical index of the mitotic activity, would compare in fertilized eggs and in eggs differentiating without cleavage. The eggs were therefore taken out of the manometer vessels and preserved in acetone until a sufficient amount of material (about 2 grams) could be collected. The thymonucleic acid content of unfertilized, activated and fertilized eggs was determined by Dische's (1930) colorimetric micromethod. Three estimations could be made. These showed that while the unfertilized eggs contained, as was expected, only traces of thymonucleic acid, the activated eggs had a content in this substance of about 0.35 milligrams per gram wet weight and the fertilized eggs of 1 milligram per gram wet weight. The thymonucleic acid synthesized in 8 hours during differentiation without cleavage amounts thus only to 30 per cent of the amount produced in the fertilized eggs during that period.

Discussion

The curve we obtained for fertilized eggs closely resembles the one published by Whitaker (1933), although its slope is somewhat steeper. In Whitaker's experiments, the unfertilized egg rate is reached by the fertilized eggs in 4½ hours and the increase over that rate at the end of 7 hours of the experiment is only 30 per cent. This difference is very likely due to the higher temperature (24.8° instead of 22°) at which our experiments were carried out: ciliary activity had just begun after 6 hours in Whitaker's case while we noticed swimming blastulae after 4½ hours. It seems therefore probable that conditions similar to those described by Tyler (1936*a*, *b*) in his work on temperature coefficients of developmental processes and cellular respiration prevail also in *Chætopterus* eggs. It is very probable that the reduced metabolic activity of the eggs differentiating without cleavage is, likewise, somehow linked with their slower development. We have already seen that the control experiments rule out the possibility that the observed difference should result from an inhibition effect of KCl on the eggs' respiration. There is also no doubt that the development of the eggs differentiating without cleavage is slowed down to a considerable extent: for instance, at the end of the experiments the fertilized eggs had turned into actively swimming larvæ while the activated eggs develop cilia only much later. Likewise, the increase of nuclear material, as indicated by the thymonucleic acid estimations, goes on at a much slower rate in the eggs differentiating without cleavage than in the fertilized ones. It is therefore likely that both the reduced oxygen uptake and the slower development are linked together; such a conclusion could support Tyler's opinion that part of the energy available in the egg is needed for the growth and differentiation processes taking place during development. Recent findings by Runnström (1933), J. Brachet (1935), Privolnev (1936), Stefanelli (1937) that the respiration undergoes cyclic changes during mitosis in eggs of different species are also in good agreement with our observations. It must be pointed out, however, that the interpretation of the results is complicated by a special factor; namely, that the activated eggs remain unicellular while the fertilized ones cleave into many cells. It is, of course, by no means impossible that oxidation processes might occur at the surfaces between the different blastomeres. Against such an interpretation, however, two facts can be cited; namely, that there is no direct relationship between the number of the blastomeres and the oxygen uptake of the egg (*cf.* Needham, 1931) and that the respiration of small marine invertebrate eggs is over a wide range independent of the O_2 tension in the surrounding medium.

SUMMARY

1. The oxygen consumption of unfertilized *Chætopterus* eggs drops in the same proportion whether they are fertilized or activated with KCl.

2. The increase in respiratory rate after that initial drop is much faster in fertilized eggs than in activated (differentiating without cleavage) eggs.

3. The lower respiratory activity of the activated eggs is linked to their special type of development.

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ON THE ENERGETICS OF DIFFERENTIATION. VII

Comparison of the Respiratory Rates of Parthenogenetic and Fertilized Urechis Eggs

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The results of these experiments show that the rise in respiratory rate that occurs during development is correlated with cleavage; eggs that fail to cleave after activation show a greatly retarded rise while those that cleave show a rise that is roughly commensurate with their division rate. Inhibiting cleavage with phenylurethane affects the respiratory rate similarly.

Theoretical Part

It has been long been known that the rate of respiration rises during development. This increase in rate is evidently not directly proportional to the increase in the number of cells (*cf.* Needham, 1931). It might, nevertheless, depend upon changes in the egg brought about by cell division, so that when cleavage fails to occur the rise in respiration would be inhibited. We have considered in previous work the dependence of the form-changes on the respiration, the rate of oxygen consumption being taken as a measure of the energy available for the various developmental processes. We consider now the possibility that the developmental changes determine in turn the rate of respiration. If, for example, early cleavage is inhibited in a manner that does not affect the absolute rate of respiration at the particular stage, then we may expect, on this basis, failure of the subsequent rise.

In the early work of Warburg (1910) it has been shown that cleavage could be suppressed in sea urchin eggs by means of phenylurethane without immediately affecting the respiratory rate. However, the question of whether or not the rate would rise later was not investigated. Also it has been shown that after parthenogenetic activation of sea urchin eggs the same increase in rate occurs that is obtained normally upon fertilization (Warburg, 1910; Loeb and Wasteneys, 1913). But here again it would be desirable to know what happens later, especially since the parthenogenetically activated eggs develop much more slowly in general and often stop in early cleavage or even fail to divide.

In *Urechis* artificial activation with a single agent may produce dividing or non-dividing eggs depending on the length of treatment. Diluted sea water (Tyler, 1931) or ammoniacal sea water (Hiraiwa and Kawamura, 1936; Tyler and Bauer, 1937) may be used for this purpose.

EXPERIMENTAL PART

The respiration measurements were made by means of the usual Warburg method using the cylindrical type of vessel previously described (Tyler, 1936) but of 18 to 20 cc. calibration volume. The quantity of eggs present in each vessel was determined at the end of the run from the Kjeldahl nitrogen, and the oxygen consumption is expressed on that basis. The quantity of eggs employed was such as to give readings of 25 to 50 mm. Brodie fluid per hour at the start of the experiment.

The non-dividing and the dividing parthenogenetic eggs were produced simply by treatment with ammoniacal sea water, as previously described. Treatments of 2 to 7 minutes with 0.01 N NH_3 in sea water give 100 per cent activation with normal polar body extrusion but no cleavage. Such eggs go through a series of monaster cycles after polar body formation, but at a slower rate than would correspond to the normal nuclear changes. After about 10 to 15 hours unicellular swimmers may develop similar to the embryos differentiating without cleavage, first described by Lillie (1902). Treatments of 12 to 17 minutes with 0.01 N NH_3 in sea water give 100 per cent activation with as much as 90 to 100 per cent cleavage. Polar body formation is usually interfered with in such eggs, as previously described (Tyler and Bauer, 1937). After 8 to 12 hours swimming embryos including some normal ones may appear.

Non-Cleaving Parthenogenetic Eggs

The results of two sets of experiments with non-cleaving activated eggs are given in Table I, along with the fertilized controls. In both the treatment was for six minutes with 0.01 N NH_3, and insemination of the control was done at the start of the parthenogenetic treatment. Activation and fertilization were 100 per cent in both these experiments. As may be seen in Table I the oxygen consumption of the parthenogenetic eggs during the first hour of measurement is very nearly the same as that of the fertilized eggs. In both experiments the parthenogenetic eggs give slightly higher values, but the difference is no greater here than the difference between the duplicate vessels. During the subsequent hours the rate rises steadily in the case of the fertilized eggs. The parthenogenetic eggs, however, show first a slight drop (second to fourth hours) followed by an increasing rate. After 8 hours the parthenogenetic eggs attain a rate that is about double their initial respiration, but only about half of that of the fertilized eggs.

The eggs from the vessels were examined at the end of the run. In both experiments the parthenogenetic eggs gave about 2 per cent of unicellular swimmers while the fertilized eggs gave 90 to 98 per cent normal top-swimming trochophores.

Cleaving Parthenogenetic Eggs

The respiration data of two sets of experiments with eggs that divide after ammonia treatment are given in Table II. In both experiments it may be seen that the respiration of the parthenogenetic eggs at the start is slightly higher than that of the fertilized controls. A third set not presented in the table shows the same initial difference, and also agrees very well throughout the run. The difference here is somewhat greater than was manifested in the experiments with the non-cleaving parthenogenetic eggs. It may be pointed out that the ammonia treatment is considerably longer here (13 to 17 minutes) and this may account for the higher initial rate.

TABLE I

Oxygen consumption of non-cleaving parthenogenetic eggs. Parthenogenetic treatment = 6 minutes with 0.01 N NH_3 in sea water. Measurements begun at 43 minutes after treatment or insemination in Experiment I and 60 minutes after in Experiment II. Values given as cu. mm. O_2 per hr. per mg. N. Temperature = 22.0° C.

Experiment	I				II		
Hour	Parth.	Parth.	Fert.	Fert.	Parth.	Fert.	Fert.
1st	4.64	4.48	4.38	4.24	4.72	4.53	4.14
2nd	4.40	4.30	4.53	4.38	3.88	5.05	4.49
3rd	4.02	3.99	5.60	5.55	3.83	5.45	5.35
4th	4.52	4.34	7.58	7.53	4.32	7.01	6.65
5th	5.22	5.07	10.01	9.93	5.56	10.10	10.20
6th	6.19	6.02	13.60	13.43	7.40	13.83	13.48
7th	6.86	7.29	16.38	16.31	9.66	18.26	17.45
8th	8.91	8.87	18.66	18.47	10.93	21.64	20.81

The subsequent readings give values that are fairly constant for the parthenogenetic eggs up to the fifth hour, while the fertilized eggs rise as usual during this time. Following this the rate rises and at the eighth hour the respiration is about half of the control rate. It is very nearly the same at that time as is obtained with the non-cleaving parthenogenetic eggs (Table I). However, differences appear later as will be shown below.

The cleavage of these parthenogenetic eggs is very slow compared with the fertilized controls. In Experiment I, at 2 hours after treatment, there were 43 per cent cleaved of which 15 per cent were in two cells, 22 in three and 6 in four. The fertilized controls were 100 per cent in four at this time. An hour later there was 75 per cent cleavage. At the end of the run (11 hours after treatment) the eggs from the vessels were 90 per cent cleaved and 30 per cent were swimmers.

In Experiment II, 60 per cent of the parthenogenetic eggs were divided at two hours after treatment, the distribution being 23 per cent in two cells, 30 in three and 7 in four. The fertilized controls were all in four cells at the time. At the end of the run (10 hours) the parthenogenetic eggs from the vessels were 95 per cent cleaved of which 5 per cent were swimmers, while the fertilized eggs gave 90 per cent swimmers.

TABLE II

Oxygen consumption of cleaving parthenogenetic eggs. Parthenogenetic treatment = 13 minutes (Experiment I) and 17 minutes (Experiment II) with 0.01 N NH_3 in sea water. Measurements began at 74 minutes after treatment or insemination in Experiment I and 53 minutes after in Experiment II. Values given as cu. mm. O_2 per hour per mg. N. Temperature = 22° C.

Experiment	I				II			
Hour	Parth.	Parth.	Fert.	Fert.	Parth.	Parth.	Fert.	Unfert.
1st	5.88	5.81	4.48	4.64	5.69	5.70	4.51	3.69
2nd	5.45	5.69	4.92	4.82	5.40	5.48	4.60	3.90
3rd	5.20	5.25	5.63	5.80	5.51	5.34	5.59	3.37
4th	5.36	5.36	7.03	6.96	5.85	5.70	6.94	——
5th	5.63	5.25	10.28	10.12	5.91	5.48	9.07	3.29
6th	5.99	5.92	14.05	13.80	7.01	6.86	13.17	3.37
7th	7.55	7.25	17.46	17.83	8.04	8.02	16.27	
8th	8.62	8.04	20.90	21.14	10.25	10.18	20.06	
9th	9.71	9.49	24.50	24.45				
10th	10.65	10.95	——	28.55				

Since the parthenogenetic eggs do not divide at all synchronously and since many may stop after one or more cleavages, the distribution becomes quite complicated. It is clear, however, that cleavage and the development of cilia are retarded.

Later Stages

Measurements on the later stages were made by culturing the eggs in dishes and washing the embryos before transfer to the vessels.

This is to avoid possible effects of bacterial growth or other changes produced in the vessels during a prolonged run. In Table III the results of one set of experiments are given. Cleaving and non-cleaving parthenogenetic eggs were prepared from the same batch along with the fertilized controls, and allowed to develop for 11 hours at room temperature (18.2° C.) before transfer to the vessels. In the fertilized lot there were more than 95 per cent top-swimming young trochophores, and only the top swimmers were transferred to the vessels. In the non-cleaving parthenogenetic lot (Parth. 5) there were no swimmers at that time. In the cleaving parthenogenetic lot (Parth. 17) 95 per cent had divided and practically all of them were bottom-swimmers.

TABLE III

Oxygen consumption of late stages of non-cleaving and cleaving parthenogenetic eggs. Parthenogenetic treatment = 5 minutes (Parth. 5) and 17 minutes (Parth. 17) with 0.01 N NH_3 in sea water. Eggs cultured for 11 hours at 18.2° before measurements were begun. Values given as cu. mm. O_2 per hour per mg. N. Temperature = 22°.

Hour	Fert.	Fert.	Parth. 5	Parth. 5	Parth. 17	Parth. 17
1st.........	21.80	22.09	12.31	12.17	11.64	11.69
2nd........	25.45	25.91	12.54	12.42	19.03	18.45
3rd........	29.38	30.48	15.11	14.90	18.74	18.96
4th........	32.39	33.32	15.01	14.65	20.75	20.26
5th........	36.17	36.18	14.44	14.15	22.60	22.35
6th........	39.94	40.48	15.76	15.72	24.76	24.98
7th........	43.31	45.70	18.98	18.75	27.70	27.95
8th........	46.50	47.70	22.18	22.73	31.73	32.67

As may be seen from Table III the two types of parthenogenetic eggs respire at very nearly the same rate at the start of the experiment. The rate is a little more than half of the fertilized rate at this time. The rate rises with time, but more rapidly in the case of the dividing eggs. At the end of the eight-hour period they are respiring at almost one and a half times the rate of the non-cleaving eggs, but still only two-thirds that of the fertilized controls. Examination of the eggs from the vessels at the end of the run showed 20 to 30 per cent bottom-swimmers (unicellular) in the case of the non-cleaving eggs (Parth. 5); 100 per cent swimmers of which 30 to 40 per cent were top-swimmers in the case of the cleaving eggs (Parth. 17); and 100 per cent top-swimmers in the case of the fertilized controls.

Fertilized Eggs in Phenylurethane

With the proper concentration of phenylurethane, cleavage may be suppressed without the initial respiratory rate being affected, as

Warburg (1910) showed on sea urchin eggs. The experiments were repeated for the purpose of covering a longer period. The results of such an experiment with *Urechis* eggs are presented in Table IV. The eggs were placed in the solution at 45 minutes after insemination, and the measurements begun 40 minutes later. As the figures in Table IV show, the initial rate of respiration is the same as in the untreated controls. The rate then rises in both, but more slowly in the treated eggs. At the end of the run their respiration is less than three-fourths of the control rate.

It is evident here that the failure of cytoplasmic cleavage is accompanied by a slower rise in respiratory rate. The rate does, however, actually rise. It may be pointed out in this connection that nuclear division goes on in the treated eggs but at a retarded rate.

TABLE IV

Oxygen consumption of phenylurethane-treated eggs. Eggs placed in 5 × 10^{-4} N phenylurethane after appearance of second polar body (45 minutes after insemination). Measurements begun at 85 minutes after insemination and 40 minutes after immersion in phenylurethane. Values given as cu. mm. O_2 per hour per mg. N. Temperature = 22.0° C.

Hour	Control	Control	Phenylur.	Phenylur.
1st	4.61	4.64	4.86	5.04
2nd	4.94	4.92	4.69	4.83
3rd	5.66	5.48	5.40	6.71
4th	6.99	7.05	6.72	5.13
5th	9.84	10.02	9.16	10.84
6th	13.34	13.54	10.80	9.26
7th	16.30	16.40	12.30	14.01
8th	18.83	18.79	13.68	14.01
9th	21.61	21.33	15.43	15.98

Runnström (1928) investigated the action of phenyl- and ethylurethane on the respiration of sea urchin eggs. The concentrations that he used gave a depression of the initial rate; nevertheless a distinct rise is manifested during the three-hour period of the experiments. He notes too that nuclear division proceeds although the respiration during the first hour may be only 35 per cent of that of the control.

DISCUSSION

The curves of Fig. 1 summarize the results. It is readily seen that the parthenogenetic eggs, although starting out at about the same rate as the fertilized eggs, do not give as rapid a rise with time. The two types of parthenogenetic eggs give approximately the same values during the early stages, but later the dividing eggs manifest a more rapid rise. If only the early period were considered this might be taken to mean that the rise in respiratory rate is not at all correlated with cleavage. However, as pointed out above, the cleavage parthenogenetic eggs divide at a retarded rate. Also, it is evident that in normal fertilized eggs no appreciable rise occurs during the early cleavage. It is therefore fairly safe to conclude that the rise in rate is linked with cleavage. But that it is not merely a matter of cytoplasmic cleavage is clear from the fact that in the non-cleaving parthenogenetic eggs and the phenylurethane-treated eggs the respiratory rate does rise with time. Here, as we have seen, nuclear division goes on and it is probably with this factor that the rise is connected.

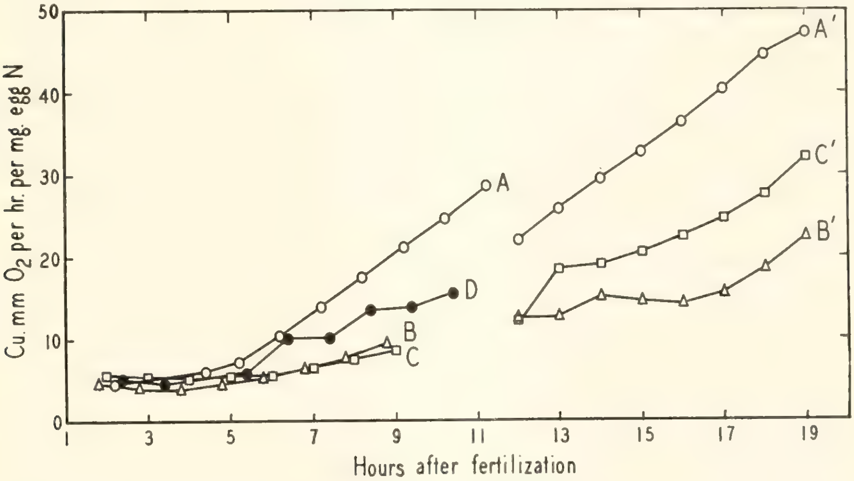

FIG. 1. Rate of oxygen consumption of *Urechis* eggs. Curves *A* and *A'*; fertilized eggs. *B* and *B'*; non-cleaving parthenogenetic eggs. *C* and *C'*; cleaving parthenogenetic eggs. *D*; phenylurethane-treated eggs. Temperature 22° C. Values are averages from all of the data. *A'*, *B'* and *C'* are not direct continuations of *A*, *B*, and *C* since the eggs had been cultured at 18.2° C. for 11 hours.

Similar experiments have recently been independently performed by Brachet (1938) on *Chætopterus* eggs that "differentiate without cleavage" and he has kindly allowed us to examine his manuscript. The results with *Chætopterus* agree very well with those presented here. In addition Brachet has investigated the nucleic acid content and finds it to be much lower in the parthenogenetic (non-cleaving) eggs than in the fertilized controls.

In experiments of this type it is, of course, important to rule out possible direct effects of the chemical agent employed. It is reasonable to assume that there has been no direct effect when the initial rate is unaltered. Such is the case in the experiments reported here with the exception of the cleaving parthenogenetic eggs (Table II) which showed a significantly high initial respiratory rate. This is very likely due to the ammonia treatment and from the subsequent values we might assume that the effect passes off. In any event, the difference would have to be greater and in the opposite direction to seriously affect the conclusion.

As was pointed out in the introduction, we might expect no rise in respiration if cleavage were blocked by some means that does not alter the absolute respiratory rate at the particular stage. But we are not dealing here with such ideal cases. In these experiments cytoplasmic division fails (non-cleaving parthenogenetic eggs) or is prevented (phenylurethane-treated eggs) while nuclear division proceeds at a retarded rate; or both cytoplasmic and nuclear division proceed at a retarded rate (cleaving parthenogenetic eggs). It is not surprising, therefore, to find that the respiratory rate does increase in these cases. That the parthenogenetic show a slower rise than the fertilized eggs is consistent with their slower development, as is also the difference between the phenylurethane-treated and the control eggs. The difference between the two types of parthenogenetic eggs may likewise be interpreted as due to differences in the rate at which comparable stages of development are reached.

Summary

1. Artificially activated eggs of *Urechis* respire at the same initial rate as do normally fertilized eggs.

2. The rate of respiration rises with time in the artificially activated eggs, but at a much slower rate than in the fertilized eggs.

3. The increase in respiratory rate with time is greater with cleaving than with non-cleaving parthenogenetic eggs.

4. Fertilized eggs in which cytoplasmic cleavage is inhibited and nuclear division retarded by means of phenylurethane give a retarded rise in respiratory rate, although the initial rate is the same as the control rate.

5. It is concluded that the delayed rise in respiration is linked with the slower development in all these cases.

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EXPERIMENTS ON LIGIA IN BERMUDA

V. Further Effects of Salts and of Heavy Sea Water

T. CUNLIFFE BARNES

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Four previous papers of this series (Barnes, 1932, 1934, 1935, 1936) have described aspects of the behavior, salt requirements and thermal range of *Ligia baudiniana*, an interesting isopod which is invading the land through the intertidal zone. The present report deals with further experiments on the capillary mechanism conveying sea water to the gills, reactions to filter paper saturated with diluted sea water, the protective action of Ca in hypotonic sea water and the combined effect of high temperature and heavy water.

Habits

It was reported previously that the release of young from the brood pouch was observed only in specimens kept submerged in sea water, but during the past summer this phenomenon occurred in a few cases in females in air over filter paper moistened with sea water. On the other hand, the molting process has occurred only in air. No adult specimens have been taken in the sea, but in rare cases an isopod will enter sea water in a terrarium to feed.

Ligia is provided with a capillary mechanism for keeping the gills moist without entering the sea. The first paper of this series stated that the uropods and spines are lowered into the water or onto a water film and the sea water then rises by this capillary path to the gills. However, Mr. M. D. Burkenroad has drawn my attention to a more important capillary conduit by which the water rises between the sixth and seventh legs to the gills and is then propelled by the gills *down* the uropods and spines (Fig. 1, *A*). The sixth and seventh leg on one or both sides may be used. Frequently the animal slides one leg over the other alternately at the start. The drainage down the uropods was described in a former paper (Barnes, 1935) in experiments in which sea water was dropped on the animals. The flow was followed in the present experiments by the addition of stains, fine particles or bubbles to the sea water. If the uropods are lowered when the last pair of legs are not drawn together, the first movement of the water is up the capillary conduit of the uropods as originally described (which may be observed by the movement of particles in the sea water). When the isopod is totally immersed, the currents produced by the beating of the gills in the surrounding sea water (indicated by the movement of particles) follow the same direction as the capillary circuit involving the rise of water between the last pair of legs (Fig. 1, B).

The sixth or seventh leg was removed in some specimens to modify the method of obtaining water. In the absence of the seventh leg a specimen standing on moist filter paper usually takes up the position for the capillary circuit via the legs (Fig. 1, *C*). It feels about frantically with the sixth leg for the missing seventh leg to complete the capillary channel. No attempt is made to use the fifth leg. Similarly if the sixth leg is removed, the conduit is impossible. Some specimens "squat" on the water film and the water rises by capillarity on the whole undersurface of the body and gills (Fig. 1, *D*). Other specimens with the sixth leg missing lower the abdomen and uropods and thus

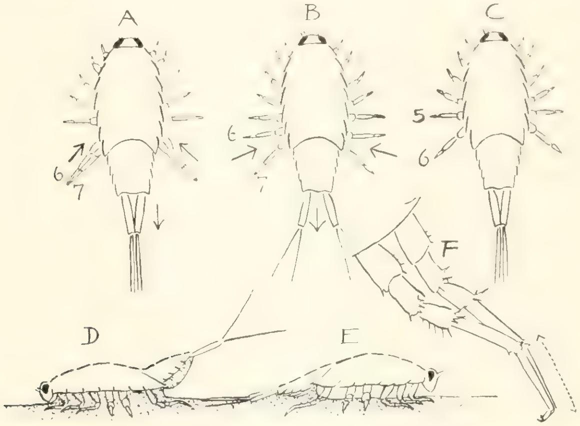

FIG. 1. Methods of moistening the gills in *Ligia*. (In these sketches the antennae and body segmentation are omitted.)

A. Specimen in air on filter paper saturated with distilled water. Water rises between the sixth and seventh leg by capillarity and is drained down between the uropods as indicated by arrows.

B. Specimen immersed in sea water. The arrows indicate the currents maintained by the beating of the gills.

C. Specimen with seventh leg removed standing on filter paper saturated with sea water. Sixth leg and uropods are in position for capillary circuit.

D. Side view of specimen with sixth leg removed standing on film of sea water (stippled).

E. Side view of specimen with sixth leg removed with abdomen lowered in film of sea water.

F. Sixth and seventh legs permanently fused along segment indicated by dotted line (found on left side of one specimen). These partially fused legs functioned normally as a capillary channel.

secure water by capillarity (Fig. 1, *E*). It is clear that there are several methods of wetting the gills. Indeed, many specimens are found which have lost the uropods and spines. These simply lower the abdomen on a damp substratum.

An interesting specimen was found in which the sixth and seventh leg were permanently fixed in the capillarity posture owing to the fusion of the last segments (Fig. 1, *F*). While walking, the double leg moved alternately with the seventh leg on the opposite side, but sometimes dragged. The joined legs functioned normally as a channel above the point of fusion.

REACTION TO FILTER PAPER SATURATED WITH DILUTED SEA WATER

Large filter papers (diameter 25″) were cut in two, one half saturated with distilled water, the other with diluted sea water and placed in a covered flat dish in a photographic darkroom having a light directly above the center of the dish. Four isopods (previously kept on seaweed moistened with sea water) were placed in the dish and their distribution on the two halves was observed at ten to fifteen-minute intervals over a period of one to two hours. The dish was rotated 90° after each reading to eliminate any unsuspected source of orientation. The animals showed a distinct tendency to collect on filter paper saturated with distilled water when the other half was moistened with sea water (Table I, and Barnes, 1935). It was found that this aversion was also shown to 75 per cent sea water, but not to dilutions of 50 per cent and below (Table I).

TABLE I

Reaction of Ligia to Filter Paper Saturated with Diluted Sea Water
(The animals were tested in groups of four)

Treatment of each half of paper	Total number of isopods found on each half	Ratio
Sea water *vs.* distilled water	69 : 123	1 : 1.78
75 per cent sea water *vs.* distilled water	27 : 73	1 : 2.74
50 per cent sea water *vs.* distilled water	93 : 96	1 : 1.03
25 per cent sea water *vs.* distilled water	109 : 73	1 : 4.9
10 per cent sea water *vs.* distilled water	27 : 21	1.28 : 1

The tendency to avoid sea water was most pronounced during the first observations in a given experiment and after an hour or more, when the paper containing distilled water was becoming dry, most specimens collected on the sea water side. Thus the ratio of specimens on the sea water side to those on the distilled water side was 1 : 3.3

for all the first readings and 2.3 : 1 for the last observation made after an average time interval of one and one half hours.

The Protective Action of Calcium in Hypotonic Sea Water

Ligia survives for only about seven hours in 100 cc. of 25 per cent sea water, but as with many other forms, the addition of calcium protects the organism from hypotonic media, apparently by decreasing permeability. As in all the experiments with solutions, individual specimens were tested in 100 cc. of solution in finger bowls. As will be seen from Table II, there is a threshold for the Ca effect (at about 12 cc. $\frac{5}{8}$ M $CaCl_2$ added to a liter of 25 per cent sea water). It is also apparent that the addition of Ca above a critical quantity (20 cc. $\frac{5}{8}$ M $CaCl_2$ added to a liter of 25 per cent sea water) has little additional protective action. It is interesting to note (see Fig. 2) that the average survival in calcified 25 per cent sea water approaches the average longevity in 100 cc. of natural sea water.

Table II

Longevity of Ligia in 25 per cent Sea Water Containing Added $CaCl_2$

cc. $\frac{5}{8}$ M $CaCl_2$ added to 1 liter 25 per cent sea water	Average longevity	Maximum longevity	Coefficients of variation	Number of specimens
	hours	*hours*		
0	7 ± 0.33	15	4.7	33
2	6.5 ± 0.20	11	3.0	30
5	7.6 ± 0.37	17	4.8	58
12	24.6 ± 3.66	119	14.8	30
15	24.1 ± 3.70	144	15.3	20
20	51.5 ± 7.98	120	15.5	20
25	37.8 ± 5.09	168	13.5	30
50	38.5 ± 3.85	180	10.0	40

The Reversibility of the Calcium Effect

Experiments were carried out to see if the protective action of calcium involves an irreversible chemical process. If so, preliminary treatment in solutions of high calcium content should lengthen the survival of isopods subsequently immersed in hypotonic sea water. However, it was found that preliminary immersion in calcified sea water from half an hour to over a day had little if any effect on the subsequent longevity of the treated specimens in 25 per cent sea water (see Table III). Likewise, preliminary treatment with sea water having a high content of sodium has no significant effect on subsequent action of hypotonic sea water. In all these tests many specimens died during the preliminary treatment so that those tested in the 25 per cent sea water represented a selected population capable of withstanding the submerged state. It must be remembered, however, that the survival of *Ligia* in any liquid medium is limited by unknown factors so that these selected specimens were already weakened by submersion. That these two factors balanced each other is indicated by the similarity of the average longevity in 25 per cent sea water of untreated and treated specimens. Thus the 152 isopods exposed to previous solutions survived 8.9 hours in 25 per cent sea water compared to the usual average of about seven hours in this medium for freshly collected individuals. In all the tests the specimens were washed by rapid immersion in distilled water after treatment in the calcium solutions, but this had no effect on the subsequent survival in 25 per cent sea water. Thus controls treated with ordinary sea water and

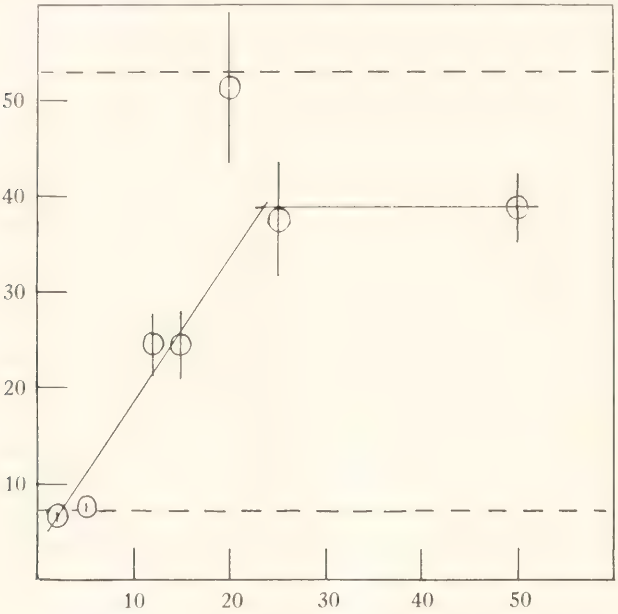

FIG. 2. Length of life of *Ligia* in 100 cc. of 25 per cent sea water with increasing amounts of $CaCl_2$.

Ordinates, average life in hours. Abscissæ, cc. of $\frac{5}{8}$ M $CaCl_2$ added to 1,000 cc. of 25 per cent sea water. The graph represents 228 tests (see Table II). Lower dotted line is the expected survival in 25 per cent sea water. Upper dotted line is the average survival in natural sea water. The vertical lines are proportional to the probable errors. The sea water average represents 265 specimens and the 25 per cent sea water average, 63 specimens. These are cumulative totals for five seasons' work. In all cases an individual specimen was tested in 100 cc. of solution.

then washed in distilled water lived the usual interval of about 7 hours in 25 per cent sea water.

Experiments in which the isopods were tested in distilled water after treatment with $CaCl_2$ give similar results (see Table IV). After exposure to $\frac{5}{8}$ M $CaCl_2$ the average survival was three hours in distilled water compared to 2.8 hours for untreated specimens.

The Combined Effects of High Temperature and Heavy Water

It was previously shown that *Ligia* succumbs rapidly in a small volume of sea water at 38° C. The following experiments were performed to ascertain if sea water containing 99 per cent heavy water would protect the organism against heat death or else potentiate with high temperature by reducing the survival time. In each case 5 cc. of sea water were evaporated and the salts redissolved in ordinary distilled water or in 99 per cent heavy water. It was found that death ensued twice as rapidly in heavy sea water at 38° C. The survival times in 5-cc. samples were 23 minutes for H_2O sea water and eleven minutes for D_2O sea water (see Table V). Owing to the scarcity of heavy water, several specimens were tested in the same sample, but no appreciable difference was observed in the survival times of the first and last isopods treated.

Table III

Longevity of Ligia in 25 per cent Sea Water after Treatment in Calcified Sea Water

Initial solution	Average length of treatment	Subsequent average longevity in 25 per cent sea water	Maximum longevity	Coefficients of variation	Number of specimens
	hours	*hours*	*hours*		
Sea water	12.2	6.2±0.30	13.5	4.8	34
50 cc. $\frac{5}{8}$ M $CaCl_2$ plus 950 cc. sea water	31.5	5.5±0.32	6.5	5.8	13
100 cc. $\frac{5}{8}$ M $CaCl_2$ plus 900 cc. sea water	21.1	6.5±0.33	9	5.0	22
200 cc. $\frac{5}{8}$ M $CaCl_2$ plus 800 cc. sea water	17.6	9.2±0.38	17	4.1	49
500 cc. $\frac{5}{8}$ M $CaCl_2$ plus 500 cc. sea water	0.5	10.5±0.39	18	3.7	40
400 cc. $\frac{5}{8}$ M NaCl plus 600 cc. sea water	25.3	9.4±0.79	25	8.4	28

Discussion

The tendency of the isopods to collect on filter paper treated with distilled water rather than on sea water paper is of interest in connection with the animals' aversion for the sea. The reaction appears to

TABLE IV

Longevity of Ligia in Distilled Water after Treatment with Calcium

Initial solution	Average length of treatment	Subsequent longevity in distilled water	Maximum longevity	Coefficients of variation	Number of specimens
	minutes	*hours*	*hours*		
None	0	2.8 ± 0.25	5.2	8.9	22
500 cc. $\frac{5}{8}$ M $CaCl_2$ plus 500 cc. sea water	33	4.5 ± 0.15	6	3.3	20
$\frac{5}{8}$ M $CaCl_2$	12	3 ± 0.20	4	6.7	12

be determined by the salt content of sea water since dilution of 50 per cent destroys the effect. It is possible that the salts on the paper stimulate the isopods to greater movement which would cause them to collect on the salt-free side. Gunn (1937) has described a hygrokinetic effect in *Porcellio scaber* whereby the greater activity of animals in dry air causes them to collect in moist locations, but this mechanism is probably not responsible for the reaction of *Ligia* described above. As was shown in a previous paper (Barnes, 1935), specimens will collect on dry filter paper when the other side of the dish contains filter paper moistened with distilled water. This refers to moist specimens. It is not known what part the flushing mechanism of the gills plays in these reactions. Bateman (1933) found that salts become slightly concentrated in the blood of specimens kept in air and it is possible that flushing the gills, especially with distilled water, enables the animal to get rid of salts concentrated by evaporation. On the other hand, specimens which have been immersed in distilled water also show the aversion for paper soaked in sea water (Barnes, 1935).

TABLE V

Longevity of Ligia in 5 cc. of Ordinary and Heavy Sea Water at High Temperature

Medium	Temperature	Average longevity	Maximum longevity	Coefficients of variation	Number of specimens
	° C.	*seconds*	*seconds*		
Sea water	38	1383 ± 45.1	2005	3.26	27
Sea water containing 99 per cent D_2O	38	660 ± 57.0	1140	8.63	11

The well-known action of Ca as a factor enabling organisms to withstand hypotonic solutions (for reference *cf.* Barnes, 1934) is strikingly illustrated by *Ligia*, which survives almost as long in 100 cc. of 25 per cent sea water of approximately 0.015 M $CaCl_2$ content as in 100 per cent of natural sea water. To the list of favorable artificial sea water solutions for *Ligia* described in previous papers may be added this new hypotonic mixture which is equivalent to sea water in which the Na, K and Mg content has been reduced to one fourth and the Ca content raised slightly. However, the *maximum* longevity in this medium is far short of the 297 hours observed in natural sea water. The very long survival of occasional specimens in sea water suggests that other factors besides salt effects are involved, such as the amount of food in the gut, the nature of the previous environment, the oxygen content, or the necessity for molting (which apparently does not occur in sea water). The high summer temperature of approximately 27° C. must also be considered.

The failure of preliminary immersion in sea water rich in calcium to protect the organism against subsequent exposure to hypotonic sea water indicates that Ca forms a loose, rapidly reversible combination with material in the plasma membrane. The existence of a threshold concentration and of a limited range in which the effect of Ca is proportional to the concentration suggests that a surface reaction is involved.

The rapid lethal action of 5 cc. of sea water at 38°, in which the ordinary water has been replaced by heavy water, was an unexpected result. The lower energy content of heavy water, which under certain conditions might be expected to protect an organism from high temperatures, was overbalanced by the well known toxic action of high concentrations of deuterium. Barbour (1937) has recently discussed the toxicology of heavy water. In larger volumes of sea water or even fresh water *Ligia* withstands high temperatures for a much longer period, probably on account of the greater quantity of oxygen present. The 5-cc. samples used in the tests were exposed to the air in a Petri dish so that the oxygen content of both heavy and ordinary sea water was probably the same.

SUMMARY

1. When presented with a "choice" between filter paper moistened with sea water or with distilled water, freshly caught specimens of *Ligia* tend to collect on the latter. Dilution of the sea water destroys this effect.

2. The survival of *Ligia* immersed in 25 per cent sea water with added calcium approaches the longevity in natural sea water.

3. Preliminary exposure of solutions of high calcium content does not protect *Ligia* from subsequent immersion in hypotonic sea water.

4. The lethal action of 5 cc. of sea water at 38° C. is enhanced by 99 per cent heavy water.

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SOME ASPECTS OF NORMAL DEVELOPMENT IN THE COLONIAL CILIATE ZOÖTHAMNIUM ALTERNANS

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Introduction

This is the first of a series of inquiries into some of the problems of organization and regulation in a colonial protozoan of a rather special type, *Zoöthamnium alternans*, whose cells collectively possess in some degree many of the attributes of an individual organism. In order to fix some standard by which to judge experimental results it was first necessary to review in detail the characteristic features of normal development in a large number of cases.

Although Claparède and Lachmann (1858) formulated a scheme of notation for cell lineage in this species, Fauré-Fremiet (1930) was the first to make a comprehensive study of its development. His primary concern was the demonstration of cytological mechanisms by which potentialities for growth and ciliospore formation were assorted to cells in characteristic positions on the colony. According to his conclusions this "cytological" factor determines the general pattern of development. His second objective, a study of time as a limiting growth factor, has no immediate bearing on the subject to be treated here. The quantitative aspects and time relations in development are to be incorporated in a subsequent report.

The data presented here augment in considerable detail the previous studies of *Zoöthamnium alternans*, particularly with regard to the features of development which have an important bearing on the problems of regulation now being investigated.

The work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summer months of 1935–6.

It is a privilege to acknowledge assistance from those whose friendly interest has sustained the author's enthusiasm over many of the dull periods of routine work.

Material

The basal portions of mature hydroids (*Pennaria* and *Tubularia*) and Bryozoa (*Bugula*) from many localities in Woods Hole harbor great numbers of attached colonies of *Zoöthamnium alternans*. The hydroid and bryozoan colonies are clean and free from the ciliates until about June 15. From then on throughout the summer the protozoa are very abundant, although their condition varies with particular habitats and with the water temperature. Grave (1933) found that the hydroids of this region are particularly healthy and free from bacterial growths, debris, etc. for the early spring months but that in mid-summer the contaminating organisms affect them adversely. This applies equally to the associated protozoa, for those obtainable in late July and August are commonly infested with parasites of several classes. The most annoying and destructive organisms are bacteria, filamentous algæ, and an intra-cellular parasite which has been identified as a suctorian (*Acineta*) by Fauré-Fremiet (1930); others of less consequence are small crustacea, diatoms, and other ciliates, e.g. *Amphileptus* and *Cothurnia*.

METHODS

The procedure for maintaining selected colonies in aquaria is an elaboration of the technique employed by Fauré-Fremiet (1930) for his study of growth in *Zoöthamnium alternans*. Hydroids bearing the protozoa were cleaned, washed, and packed into large glass vessels of running sea water. Ruled and numbered slides were placed face down on the infected hydroid material and left for about 12 hours. They were then removed and washed by passing under a stream of running water. The precautions taken at these stages to remove adhering scum, debris, algæ, other protozoa, and minute crustacea, determined to a great extent the age to which the colonies could be grown.

Some of the mature asexual migratory cells, the ciliospores (Wesenberg-Lund, 1925), liberated from the "wild" colonies during the 12-hour interval, attached themselves to the ruled surface of the numbered slides. Slides were examined in Petri dishes, the positions of the recently affixed ciliospores recorded, and then transferred from the Petri dish to correspondingly numbered slots (vertical) in wooden racks, immersed in a 15-gallon aquarium. Cultural conditions were very much improved by the use of filtered sea water. Two 2-liter aspirator bottles packed with sand and stoppered with glass wool proved to be adequate for the purpose. When cleaned daily their combined outputs averaged about 50 gallons per hour.

Routine counts were made with a 4 mm. dry objective. When optical sections of the barely immersed colonies were not clear, the water level in the Petri dish was adjusted by a slight turn of the dish on the slanted microscope stage until the surface film flattened the colony. This was done as a last resort, for contact with the surface film elicited rapid contractile responses, frequently resulting in the loss of important zoöids.

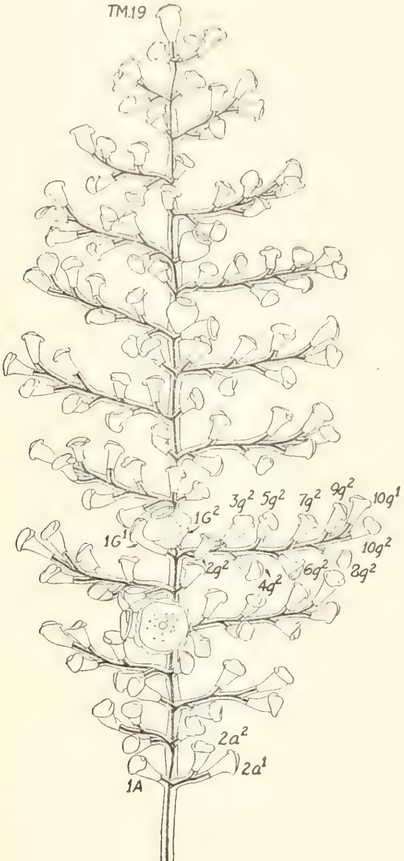

Fig. 1. A relatively mature colony of *Zoöthamnium alternans* showing the characteristic arrangement of the zoöids and branches. Axial microzoöids on branches *E*, *G*, *L*, and *N* are differentiating into ciliospores; one of them at *E* is in an advanced stage of metamorphosis, almost ready to break away from the parent colony. Approximately × 100.

General Form of the Mature Colony

The tapering frond-like configuration of the older expanded colonies is shown in Fig. 1. Its branches project obliquely from the perpendicular axis. They lie in the same plane but alternate on right and left sides at successive nodes. The zoöids of the branches occur singly for the most part, likewise alternating in position along the branch axis. While the branches of this species usually consist only of a single axis, in rare cases the zoöids may divide one or more times to produce branches of the second order.

The expanded colony is essentially asymmetrical, i.e. characterized by three heteropolar axes. The primary axis is firmly cemented to the substrate at the basal end and bears the actively dividing terminal macrozoöid (*TM.*) at the apex. The lateral axis is heteropolar by virtue of the alternating branches, each one terminating in a single cell, the dominant branch zoöid. "Anterior" and "posterior" surfaces (Fauré-Fremiet, 1930) are defined by the curvature of the branches as well as by the position of the zoöids: the branches curve anteriorly while the zoöids are inclined posteriorly (toward the convex surface). A sample count showed that the first branch occurred nine times on the left side to four on the right. Both dexio- and leiotropic colonies were reported in another species, *Zoöthamnium arbuscula*, by Furssenko (1929).

Beginning at a point about mid-way between the basal (attached) end of the stalk and the first branch, a heavy contractile central cordon, or "neuro-muscular" cord, within the stalk substance ramifies throughout the colony (Fig. 1). Its branches terminate distally at the basal ends of the zoöids. It is thus continuous from branch to branch and from cell to cell. More will be said about the importance of this structure in a later section.

NORMAL DEVELOPMENT

The scheme used to designate the lineage of the zoöids is a modified form of Fauré-Fremiet's adaptation of the original rule formulated by Claparède and Lachmann (1858–60), who were the first to note development in this species according to a definitely determined pattern.

The mode of propagation in *Zoöthamnium alternans* is almost exclusively asexual. Axillary branch zoöids at various levels in larger colonies are transformed into migratory ciliospores. These liberated motile individuals are comparatively large cells, metamorphosed zoöids, whose sub-conical bodies flatten antero-posteriorly into cells having a thickened bi-convex lens-like appearance. Their rapid swimming or creeping movements are effected by means of a heavy equatorial girdlet of cilia. The oral disc and peristomal cilia are retracted and probably undergo reorganization during the motile phase.

After a migratory existence of several hours duration the ciliospores become relatively quiescent, hovering about within a limited radius with aboral end in contact with the substrate, finally fixing themselves to the surface by means of a scopula. The secretion of the stalk begins; the girdlet of cilia becomes inactive and disappears as the peduncle elongates. The lens-shaped ciliospore then begins to assume the appearance of a very large sub-conical zoöid with retracted adoral zone. The first section of the peduncle secreted is a fairly heavy, non-contractile cylinder with thickened cuticle and homogeneous hyaline medullary substance. At some point between 200–300μ the secretory activity of the ciliospore is altered: the continuity of the secretory process is interrupted, the basal end of the cell increases in diameter, and the formation of the neuro-muscular cord begins. This node marks the lower limit of stalk contractility. The contractile cord is an integral part of the stalk structure from this point onward.

The division of the initial individual occurs approximately 15 hours after attachment. The first division furrow defines the median plane of the future colony. Succeeding furrows bear a definite and constant relation to the first, in such a manner that the daughter cells occur first on one side of the mid-line then on the other. The two daughter cells resulting from the first division are always markedly unequal in size (Fig. 2). The large daughter remains apical (terminal) in position.

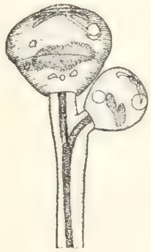

FIG. 2. Developing colony at the 2-cell stage. Drawn from a living specimen in which the macronuclei were faintly visible. × 250.

In the current terminology it is designated as the terminal macrozoöid No. 1. The small lateral daughter, the first cell of the first branch, is characterized as the median microzoöid *A*.

Dichotomous branching of the stalk occurs when the secretory basal portion of the cell constricts during the fission process. With the onset of secretory activity (i.e. stalk formation) the sister cells gradually move away from the point of origin. Material secreted by the terminal macrozoöid prolongs the axial stalk. The branch cell produces a somewhat smaller stalk extending laterally from the junction.

The apical cell (*TM.* No. 1) divides again at the second node in approximately 8 hours, producing an apical cell of the second generation (*TM.* No. 2) and a median microzoöid *B*. This time, however, the branch cell lies on the opposite side of the axis from the previously produced microzoöid *A*. Repeated divisions of the apical cell extend the principal axis of the colony, each time similarly producing a terminal macrozoöid of the succeeding generation (3, 4, 5, . . .) and an alternating branch zoöid of a higher level (*C*, *D*, *E*, . . .). The position of successive branch microzoöids is strictly alternative. Colonies with as many as 25 branches were frequently observed on the culture slides but it is doubtful that this represents maximum development. One colony produced 33 branches before succumbing to external parasites.

Following the first division of the apical cell there is a gradual reduction in the size inequality of the resulting daughters until, after 8 to 10 generations of axial development, or in some cases even fewer, their volumes immediately after a mitotic period are approximately equal. For experimental purposes it is essential to distinguish between recently formed daughters of later generations along the main axis. To this end it is necessary to refer back to the previous generation. If the left-hand cell of that generation is differentiating into a branch cell, then the right-hand position of the undifferentiated cell at the new mode may be taken as assurance of its presumptive branch relations. Likewise position is the chief diagnostic criterion for the identification of undifferentiated branch zoöids.

The generalized pattern of branch development given below concerns only the position of the cells produced, for as will be seen later, the descendants of the median microzoöids are not always equivalent in their prospective values. The symbol X (or x) is used to denote branch generations in general (Fig. 3).

Lateral extension of the stalk bearing the median microzoöid X continues until the cell divides. Microzoöid X gives rise to two structurally similar cells, microzoöids $1X$ and $1x$. Microzoöid $1X$ occupies a median position with respect to the main axis of the colony, whereas $1x$ is the lateral member. Actually $1x$ immediately assumes the terminal position on the branch axis, thus constituting the growing point of the branch. Marked differential behavior now characterizes the two sisters: $1X$ lays down a short segment of stalk and then remains quiescent for some time; on the other hand, $1x$ actively extends the branch axis, then divides again within a few hours. Microzoöid $1x$ then produces $2x^1$ (median) and $2x^2$ (lateral). Cell $2x^2$ remains behind as a common nutritive branch zoöid while $2x^1$ assumes the terminal position and continues as before, giving origin to $3x^1$ (lateral) and $3x^2$ (median); $3x^1$ to $4x^1$ and $4x^2$, etc. Each time the alternate secondary daughter is left behind as a common zoöid (Fig. 1).

Further mitotic activity in the common branch zoöids $2x^2$, $3x^2$, $4x^2$, etc. was not characteristic of the normal colonies whose histories were charted. About 2 per cent of the total number of colonies under surveillance showed a tendency toward precocious development. In these cases the repeated divisions of all branch zoöids led to such complex formations that the lineage soon became impossible to follow.

Conversely, microzoöid $1X$ on branches up to about level J commonly divides once, forming $1X^1$ (median) and $1X^2$ (lateral). Its mitotic activity on more distal branches is infrequent. On several branches of each colony the cell $1X$ or its descendants metamorphose into the migratory ciliospores which break away from the parent colony, leaving behind a short, stubby peduncle. Occasionally $1X$ produces a second or sometimes a third generation thereby augmenting the number of propagatory zoöids.

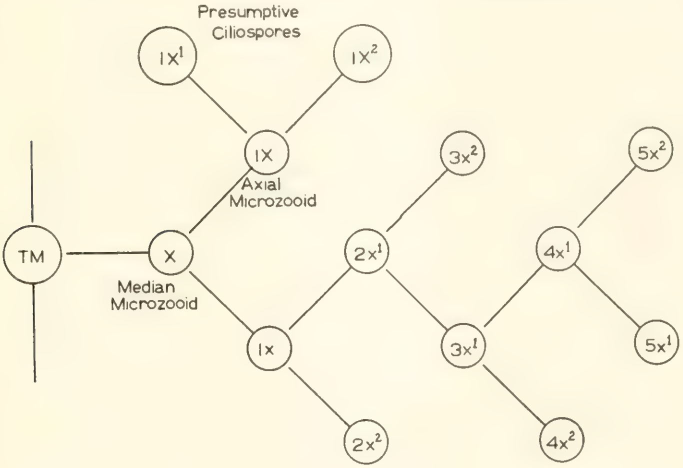

FIG. 3. Schematic representation of branch development.

DIFFERENTIATION

The distribution of the heteromorphic zoöids to be described is a generalized account based upon a tabular evaluation of 200 cases of normal and experimentally cut colonies (proximal portions only), many of which were lost before complete development. Inclusion of the basal parts of operated colonies in the tabulations seems to be justified since statistical summaries of both normal and operated colonies indicate no consistent variation as regards either rate of development or position of the differentiating zoöids.

During the life history of a colony six types of zoöids may be formed although not all of them are apt to be present at any one time.

1. The *common nutritive microzoöids* constitute the majority of the colony cells. They are the trumpet-shaped individuals characteristic of the genus. Notwithstanding the fact that most of them never assume any other form, they may be regarded as generalized components of prime colonies because (*a*) under undisturbed laboratory conditions some of them at random positions along the branch axis metamorphose into microgamonts, and (*b*) experiments may be performed in which variously situated common zoöids may be induced to differentiate into actively reproducing apical cells. Their capacity to differentiate into another type under imposed conditions gradually diminishes in time so that in older colonies the "vegetative" function becomes fixed. They can then be considered as somewhat specialized zoöids. The shape of zoöids that have persisted for relatively long periods of time without division are decidedly more slender and elongate than the "average" zoöids. This appears to be a modification of form rather than an alteration of volume. The extension of the protoplasmic body as well as its peduncle also appears to be gradual and continuous subsequent to the post-mitotic growth period.

2. Each colony bears a single *terminal macrozoöid* (*TM.*) at the apex of the principal axis (Figs. 1 and 2). In the earlier stages of colony formation three structural peculiarities clearly distinguish it from the common zoöids. It is considerably larger than any of the common types for the first ten or more axial generations, the body is characteristically flexed, and numerous annular striations in the pellicle are very pronounced. After ten generations or so its form individuality progressively diminishes. At levels beyond the twentieth branch only its position and generative activity are distinctive.

3. The *terminal branch zoöids* ($1x$, $2x^1$, $3x^1$, . . .) are almost diminutive replicas of the terminal macrozoöid. For the first few generations they are identified shortly after dividing by fairly marked pellicular annulations and a moderate anterior flexure of the tapering body. Near the close of branch development their patent features are obscure. Standard criteria of position and, to some extent, proclivity toward more rapid stalk formation and division must be relied upon to distinguish them from their common neighbors, the secondary daughters. Finally, at the terminus of a fully formed branch the last pair of zoöids are usually indistinguishable. This point represents about four generations on branch *A*, ten at intermediate levels (e.g. *II*), and progressively fewer after that (Fig. 1).

4. The most striking of the heteromorphic zoöids are the immature *ciliospores*, or asexual propagative cells which represent differentiated microzoöids of the order $1X$ or descendants. It is important for later discussion to note that the function of ciliospore formation is normally but not invariably restricted to these microzoöids. They have been observed to arise from microzoöids $5d^2$ and $6d^2$ in one instance, and $10h^1$ and $10h^2$ in another. Fauré-Fremiet (1930) found that the terminal macrozoöid was sometimes transformed into a migrating ciliospore but, unlike the usual ciliospore-forming cells, was unaccompanied by an endomictic reorganization.

Prior to ciliospore formation microzoöid $1X$ undergoes a profound growth in size. Very accentuated concentrically disposed pellicular furrows appear during the growth process. It is impossible to predict whether or not it will divide, for in a small percentage of the cases it is transformed directly into a motile ciliospore thereby terminating the lineage of that cell on the colony. Division of $1X$ in from 20 to 70 hours after derivation from the median microzoöid X is the rule, however. It is noteworthy, perhaps, that fission may occur after the onset of differentiation. The increased volume at this time is considerably in excess of the pre-divisional growth of the other zoöids. The two descendants, $1X^1$ (median) and $1X^2$ (lateral), are seldom equivalent in their size relations; the lateral daughter is usually the larger and the first to metamorphose.

Complete differentiation into motile cells already characterized involves the appearance of a girdle of long, closely set cilia about the equatorial region, the accentuation of the annulations, introversion of the oral disc, and differential growth in the plane of the diameter, eventually flattening the zoöids into disc-shaped bodies. It is believed that they are able to feed to some extent when the oral disc is introverted and the adoral zone folded. The gullet remains open and the undulating membrane within persists in its activity. The final metamorphosis of $1X^1$ may be deferred until a second generation of potential ciliospores ($1X^{11}$ and $1X^{12}$) are produced. The origin of at least four ciliospores from some axial microzoöids has been observed. Whether or not they represent three generations of alternate development or two generations formed dichotomously is still an open question.

The production of ciliospores is restricted to a limited number of loci on any given colony. Commonly there are but three or four such loci on one colony, although one protocol shows as many as seven. A generalized colony as visualized from tabulated data bears florescences at levels *D-K-P-T-AA*. They have been recorded for all nodes along the axis between B to W inclusive, but never at consecutive nodes in any one case. It should also be stated that $1X$ on many branches divides without giving rise to ciliospores. Its descendants persist as common microzoöids.

5. *Microgamonts* (*microgonidia*, Wesenberg-Lund, 1925; *microconjugants*, Furssenko, 1929) develop from otherwise visibly undifferentiated microzoöids at the terminal or sub-terminal position along branch axes. The observed time interval between the formation of the microzoöids and their complete transformation into mature, liberated swimmers varies between the limits of six to thirty-six hours. The first sign of approaching metamorphosis is the appearance of a faint furrow about the equatorial region of the tapered body. The zoöid is thus marked into a larger peristomal portion and a smaller basal portion. Cilia gradually grow out from the body at the furrow. At first very short, they soon develop into a broad girdle whose movements simulate those of a slowly waving undulating membrane (Fig. 4). Meanwhile the diameter of the cell increases. The oral half containing the retracted peristomal disc is transformed into the slightly flat, sub-conical oral end of the swimmer. The posterior portion is further reduced to a very slightly convex aboral surface at whose center the stalk is still attached. Its struggling movements gradually accelerate until at full maturity it breaks away from the parent colony. At full term the microgamonts resemble ciliospores in their eccentric body outline, ciliature, and general movements. They are, however, not perceptibly larger than the common zoöids, show but faint indications of annulations, and swim more rapidly.

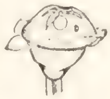

FIG. 4. Differentiating microgamont about 5 hours before the completion of metamorphosis. × 250.

While cytological studies were not attempted, it is definitely established that these are the migratory members of the copulatory series. When liberated some have been observed to swim rapidly back and forth in an arc about the parent colony, occasionally coming to rest on some zoöid, or crawling about on the branches and over the individuals in various positions, and eventually swimming away as though its relatives were not receptive of its attentions. The duration of the migratory phase was not determined. One was observed to settle upon terminal macrozoöid No. 3 of a young colony possessing nine cells in all. Two and one-half hours later the aboral end of the migrant was firmly fused with the lateral surface of the apical cell. By that time the ciliary crown of the swimmer had been resorbed.

As regards origin, it is frequently difficult to determine if microgamonts arise from prospective terminal or sub-terminal branch microzoöids because of the facility with which the prospective values of very recently produced daughter cells may be altered. For example, if the terminal cell (e.g. $2x^1$) is accidentally lost, the sub-terminal cell ($2x^2$) immediately assumes the terminal functions. If loss occurs soon after fission, when both daughters are superficially alike, the stalk remnant left behind at the node occurs in the alternate position, whereas the cell which normally would have been the alternate ($2x^2$) becomes the growing point. The tendency of the terminal branch cell to differentiate into a microgamont is conclusively demonstrated by the successive metamorphosis of sister cells. This means that the continued lateral growth of a branch subsequent to the production of a microgamont may represent each time an early (undetected) transformation of the secondary zoöid into a new terminal cell. The confusion is apt to arise because the alternate position of the stubby stalk left by the lost cell or by the migrating microgamont cannot always be diagnosed accurately. Fortunately in many instances the terminal branch cell is well indicated either by position or by symptoms of an approaching division by the time the alternate cell shows signs of becoming a microgamont.

Tabulations show that 85 per cent of the microgamonts observed on colonies whose complete developments were charted, were produced by the four basal branches A, B, C, and D, enumerated in decreasing order of importance. The rest arose from zoöids on branches E, F, G, and K. They were formed either by the terminal ($-x^1$) or alternate ($-x^2$) cells as far out on the branches as the sixth generation on A, B, and C (including $5a^{21}$ and $5a^{22}$), the seventh generation on D, and the eighth generation on F. None were observed to come from the median microzoöid X, the axial microzoöid $1X$ or its descendants.

The majority of the colonies bore but one or two microgamonts; a few produced as many as nine but not more than three for any one branch. They occurred simultaneously on different branches but successively from the alternate zoöids on any given branch. And finally, it is important that they differentiated only from branch zoöids at or near the terminal position, never from the more axial (older) microzoöids.

6. *Macrogamonts* were found only in the terminal macrozoöid position on the primary axis. Attempts to discover some morphological or behavioral distinction between terminal macrozoöids and pre-

conjugant macrogamonts were negative, but this possibility is not precluded since of the three or four hundred colonies inspected, none may have happened to have been preparing for conjugation. The presence of an attached microgamont constituted the sole criterion for identification.

Conjugants at all levels between the terminal macrozoöid positions three to twenty-four inclusive were observed. The youngest colony possessed but nine cells in all, including the conjugant. Two successive conjugants, five generations apart, were recorded for one colony.

The incidence of conjugation reached a maximum during the month of July; before or after that the conjugants were quite rare. Even in July only about 5 per cent of the colonies bore conjugants although more of them were producing microgamonts. Many of the colonies produced no gamonts whatever during the period of development on the slides.

Some of the effects of conjugation upon colonial development will be treated in a later section.

REACTIVE CAPACITIES

The motor responses of *Zoöthamnium alternans* are developed to a relatively high degree. Very local reactions by individual zoöids result in the retraction of the ciliated oral disc. More general responses evoked by altered salinity of the water, sudden contact stimuli, etc., are expressed by an extremely rapid contraction of the stalk and the zoöids. Gradual relaxation or extension ensues within fifteen to thirty seconds unless the stimulus is sustained. This manifestation of irritability constitutes one of the major technical difficulties for experimental work. When the colony is contracted, it is practically impossible to distinguish a given zoöid or to cut the stalk at a desired point. Fortunately for the experimental work the stalk alone may be touched gently without disturbing the colony. A series of attempts to inactivate the colonies by subjecting them to a variety of narcotics in various concentrations were quite unsuccessful. Freezing temperatures did not modify the contractions to any great extent.

SUMMARY

1. The frond-like colonies of *Zoöthamnium alternans* develop according to a well-determined pattern. This development is described with particular reference to the origin of the heteromorphic zoöids.

2. An asexual propagative zoöid (ciliospore) detaches from a mother colony, swims away, and settles down to form a new colony. When affixed to the substrate the ciliospore begins to secrete a peduncle or

stalk. The first portion of the peduncle is flexible but not contractile. From a point some 200 to 300μ above the hold-fast, the ciliospore suddenly begins to produce a contractile cord in the core of the hyaline stalk substance. Distal to this point the contractile cord is an integral part of the stalk; it is continuous from branch to branch and from cell to cell.

3. When the stalk is approximately 500μ long the ciliospore divides unequally. The larger daughter remains axial in position. It represents the first generation of the terminal macrozoöid series (*TM.* No. 1). The smaller lateral cell is the initial zoöid of the first branch, the median microzoöid *A*.

4. Successive divisions of the terminal macrozoöid produce each time a terminal macrozoöid of the next generation and a median microzoöid. The latter are strictly alternate in position: they alternate on right and left sides of the primary axis at successive nodes. As many as thirty-three generations of the terminal macrozoöid have been observed.

5. The first division of the initial branch zoöid gives rise to an axial microzoöid and a lateral stem cell. The stem cell generates alternating zoöids of the main branch strain whereas the axial microzoöids on some of the branches represent the presumptive ciliospores.

6. At least four types of zoöids comprise a colony: (1) a single terminal macrozoöid at the apex of the primary axis, (2) many common microzoöids scattered along each branch axis, (3) a terminal zoöid at the tip of each branch, and (4) immature ciliospores which arise from axial microzoöids on some of the branches. During epidemics of conjugation two more types may be formed: the terminal macrozoöid may be transformed into a macrogamont, and common microzoöids at random positions may metamorphose into migratory microgamonts. The spatial distribution of the heteromorphic zoöids is described in some detail.

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FORM REGULATION IN ZOÖTHAMNIUM ALTERNANS

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Introduction

Interest in morphogenetic studies has long centered about the part-whole relationships obtaining throughout the formative period in organic development. In so far as a system comprising several parts is concerned, the term *organization* implies the existence of integrating factors that condition to some extent the limits and direction of regional specialization. A remarkable number of investigations on metazoan organizers have already demonstrated the importance of extrinsic factors upon determination in specific parts. It was felt that additional information about these factors could be gained by applying operative techniques to an animal type in which, presumably, the interrelationships have not attained so great a degree of complexity.

The principal endeavor of this work was to investigate some of the qualitative and quantitative aspects of growth and differentiation in *Zoöthamnium* colonies, regulating after the removal of actively growing (distal) parts, and to compare regulative behavior with the normal development already described by Fauré-Fremiet (1930) and Summers (1938). The results have made it possible to offer a rough map of potencies and prospective values of individual cells at various positions in the colonial pattern and to indicate some of the changes in the expression of inherent potencies which may be induced by experimental means.

It is a pleasure to thank Professor L. R. Cleveland of Harvard University for extending to the author the privileges of his laboratory during January, 1937.

Materials and Methods

The materials and techniques used in this study are similar to those described in the previous paper and need not be repeated here. The only additional detail pertains to the method of shearing the stalk. Two fine scalpels made from No. 9 sewing needles were used for the purpose. One was brought to rest against the surface of the stalk and the other sheared against it in scissors fashion.

The plan of attack is by no means new, but the type of organization dealt with seems to promise a fresh approach to the current problems of form determination. A colony of *Zoöthamnium alternans* is admirably adapted to work of this kind by virtue of the regularity and precision with which the characteristic colonial pattern develops. The alternating arrangement of the branches and cells makes it comparatively easy to follow the history of any one cell throughout the course of its development for evidences of growth, division, or differentiation. The spatial relationship of the cells minimizes to a great extent some of the factors so difficult to evaluate for compact tissues. Crowding effects such as mutual contact, pressure, etc. (Peebles, 1931) are of no great concern here. Then, too, the separated cells are uniformly bathed by an almost constant medium, filtered sea water. Physiological relations between them are effected through a well-defined channel, the stalk with its neuro-muscular cord (Fauré-Fremiet's "cordon central"), rather than through the general expanse of juxtaposed cell membranes.

REGULATIVE DEVELOPMENT

Standards of Judgment.—Colonies maintained for several days on slides are apt to be attacked by internal parasites or covered by plant growths of one kind or another, especially in the basal regions. When operations are made the axial growth of a colony is retarded for an average of 23.1 hours pending the formation of a new terminal macrozoöid. It is during this period of arrested development that adverse environmental conditions are liable to bring about an incapacitation or loss of important zoöids before a decision relative to the success of the operation can be reached. A small proportion of the successful operations shown in Tables I and II do not appear in subsequent tables because they were destroyed or abandoned after indubitable signs of new terminal macrozoöid differentiation had appeared but before descendants were produced. In the absence of a regenerate, the responses were recorded only when all of the structural characteristics of the new terminal macrozoöid were established and, in addition, the "activated" branch developed an anterior flexure. In consequence of the stalk curvature the new terminal macrozoöid assumes the apical position upon an anteriorly directed axial stalk. The point of curvature marks the node (Figs. 3 and 5) at which the stalk suddenly increases to a diameter approaching that of the original axis.

Regenerative responses were arbitrarily called negative only when one of the following conditions were realized: (*a*) there was no activity for at least 48 hours; (*b*) in the event that mitotic activity continued for a generation or two, a minimum of 72 hours was allowed for signs of regulatory activity; (*c*) when the terminal branch zoöid in the line of succession metamorphosed into a migrating zoöid of some kind. The thirty-seven negative cases shown in Tables I and II were maintained for a mean time of 94.3 hours after the last division, with extremes of 52 to 212 hours.

TABLE I

		PARTS CUT AWAY								
		TM+0	TM+1	TM+2	TM+3	TM+4	TM+5	TM+6	TM+7	Total
LEVEL OF OPERATION	Q	1								1
	P	(1)	(2)		(1)					(4)
	O	2 (1)	1 (1)	(1)	(1)					3 (4)
	N		(1)							(1)
	M	1 (1)	(1)							1 (2)
	L	(1)	(1)							(2)
	K	1		(1)	(1)					1 (2)
	J	2 (1)								2 (1)
	I		1	1	1	(1)				3 (1)
	H	1	(1)				(1)			1 (2)
	G	3 (1)	1 (1)	(1)	(1)					4 (4)
	F	4	1							5
	E	1	1		1					3
	D	3	2							5
	C	6	4		1	1				12
	B	3 (1)	4 (1)	2		1		(1)		10 (3)
	A	9 (6)	6 (3)	6	2 (2)	1	1	1	1	27 (11)
Total		37 (13)	21 (12)	9 (3)	5 (6)	3 (1)	1 (1)	1 (1)	1	78 (37)

The distribution of regulative responses in 115 operated colonies summarized according to the number of apical branches cut away: *T.M.* + 0 = only the terminal macrozoöid removed; *T.M.* + 1, *T.M.* + 2, etc. = terminal macrozoöid plus one, two, or more branches removed. The letters in the left-hand column designate the branches from which the regenerates arose. The numbers of cases in which no response occurred are shown in parentheses. The first responses of the successively operated colonies are included. No attempt has been made to indicate the number of zoöids on the branches dissected away; it may be estimated by referring to Fig. 1 in the previous paper.

All colonies were abandoned when the important zoöids appeared to be unhealthy.

Adjustment of Descriptive Notation.—An arbitrary departure from the standard notation (see Fauré-Fremiet, 1930; or Summers, 1938) seems to be advisable in view of the complications arising from the designation of generations produced by the secondary zoöid near the point of origin of the new terminal macrozoöid. To illustrate: if the new terminal macrozoöid differentiates directly from $2a^1$, then the secondary zoöid $2a^2$ continues to generate zoöids of the branch *A*, viz. $2a^{21}$, $2a^{22}$; $2a^{211}$, $2a^{212}$, etc., which complicates a system already difficult to summarize briefly.

At this point it is proposed to adjust the terminology so that the new terminal macrozoöid (actually $2a^1$) corresponds in position to the original terminal macrozoöid (*T. M.* No. 1), but is distinguished from it by a prime (′) symbol. The lateral branch zoöid $2a^2$ becomes *A′*, the first zoöid of a new branch at the original *A* level (Fig. 1).

This adjustment is valid if subsequent products are to be treated descriptively as new primary branches rather than as branches of the second order on the first branch below the cut. This is nothing more than a manipulation of terms to facilitate comparison of normal and regulating colonies.

TABLE II

		ORIGIN OF NEW TERMINAL MACROZOOID										
		1X	$1X^1$	1x	$2x^1$	$2x^2$	$3x^1$	$4x^1$	$4x^2$	$5x^1$	$6x^1$	Total
LEVEL OF OPERATION	Q			1								1
	P			(1)	(1)		(1)			(1)		(4)
	O	2			1 (1)		(2)	(1)				3 (4)
	N						(1)					(1)
	M			(1)	1		(1)					1 (2)
	L				(1)					(1)		(2)
	K			1				(1)			(1)	1 (2)
	J			2	(1)							2 (1)
	I	1	1			1			(1)			3 (1)
	H						1		(1)	(1)		1 (2)
	G		1	2	1 (2)	(1)				(1)		4 (4)
	F			2	2	1						5
	E	1			1			1				3
	D			1	3			1				5
	C			8	3		1					12
	B			5 (2)	2	1	1	1	(1)			10 (3)
	A	1 (1)	(1)	15 (7)	5	(1)	5 (1)	1				27 (11)
Total		5 (1)	2 (1)	37 (11)	19 (6)	3 (2)	8 (6)	4 (2)	(3)	(4)	(1)	78 (37)

Lateral distribution of operations. This table summarizes the data from Table I according to regulation by branch generations irrespective of the amount of colony cut away. The left-hand column represents the branches from which the regenerates developed; headings indicate the position and generation of the zoöids which produced the new terminal macrozoöid. The negative responses are shown in parentheses.

Trauma.—For a number of reasons it appears probable that traumatic shock effects are not significant factors in post-operative adjustments of colonial form. Cells adjacent to the cut areas and elsewhere soon expand and feed as before. Processes of mitosis or differentiation, in progress at the time of operation, continue without perceptible interruption. Or these processes may begin at varying intervals after cutting, in any of the branch zoöids, except that which becomes the new terminal macrozoöid. Furthermore, when there is

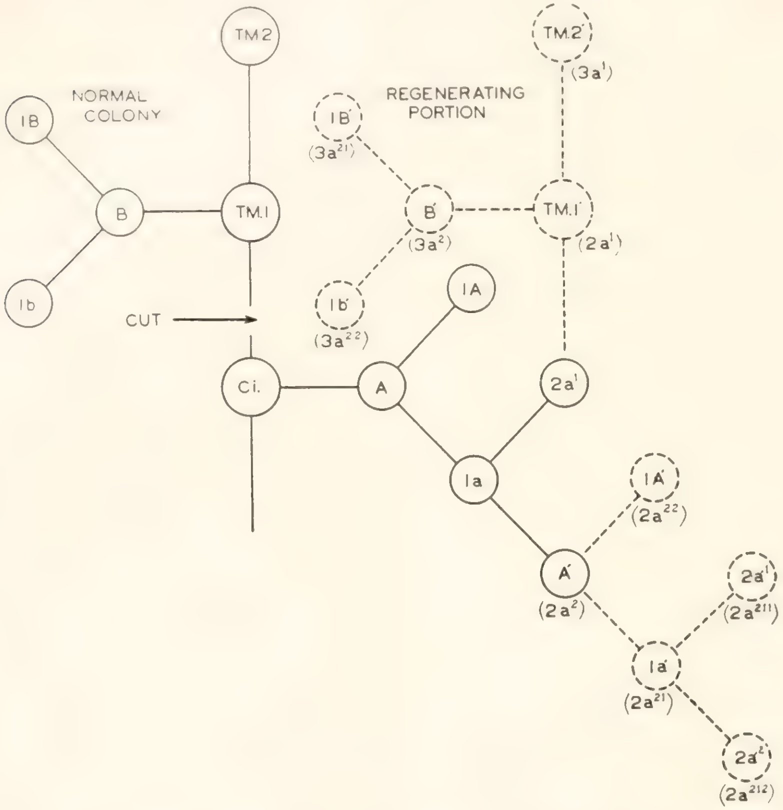

FIG. 1. Diagram of regulative development. The revised notation for designating cell lineage in regulating portions is indicated within the circles. The symbols in parentheses under the circles illustrate how cumbersome the conventional terminology would soon become if applied to the regenerates. The diagram shows the lineage of a regenerate from branch *A*. If the principal axis is severed between branches *A* and *B* at a time when there are three cells on branch *A*, the regenerate usually arises from the terminal branch zoöid $2a^1$ as illustrated. The sub-terminal zoöid ($2a^2$) assumes the terminal branch position A' and continues to generate branch cells. The new branch B' is produced by the first division of the new terminal macrozoöid $T.M.\ 1'$. The parts produced after cutting are drawn with broken lines.

no regulation, or when the median microzoöid divides one or more times before the new terminal macrozoöid is recognizable, the first branch below the cut continues to generate common zoöids as before. Relative to division rates, the available data indicate that, exclusive of the one which bears the presumptive terminal macrozoöid, branch growth is not perceptibly altered after cutting. As a rule there is a lag in the development of the activated branch pending the differentiation of a new growing point.

The only effects of mechanical disturbance are evidenced for a very short time after cutting by a state of irritability during which the contraction of decapitated colonies is frequent, irregular, and sometimes tetanic. But normal overt behavior is resumed within a few moments when the stalks are shorn cleanly at internodal points. Operations were considered acceptable only when the normal reactions were regained within a relatively short time. Cases where only the neuro-muscular cord of the stalk was injured are to be treated in another section.

Distribution of Operations.—A general résumé of the experimental results in terms of initial regulative responses in *Zoöthamnium alternans* is given in Tables I and II. Of the 144 protocols at hand (acceptable operations), 78 yielded positive responses and 37 were negative; the remainder were inconclusive according to the standards chosen and are omitted in the digests.

Table I summarizes the responses to various types of cuts made at the several levels along the principal axis irrespective of the number of generations on the regulating branches. In Table II the same protocols are tabulated according to regulative responses by the various branch generations without regard to the number of branches or zoöids removed. In this table the negative cases show only the zoöids which were expected to reconstitute the axial growing point; some of these, failing to regulate, continued to develop laterally without further differentiation.

The symbols X and x are used to indicate generations on a generalized branch (Summers, 1938). With reference to a specific branch, e.g. branch D, the symbol $1x$ refers to the microzoöid $1d$, and $1X$ means axial microzoöid $1D$.

Simple Cut-offs.—In general when the terminal macrozoöid was cut off, the terminal cell of the first branch below the cut differentiated into a new, well-defined terminal macrozoöid whose first and subsequent divisions proliferated the alternating median microzoöids (initial branch cells) of the regenerate. Some operations were made at a time when a single cell, the median microzoöid X, represented the adjacent rudimentary branch. In all such instances recorded at least one division followed without perceptible delay, thus producing an axial microzoöid $1X$ and the presumptive terminal macrozoöid $1x$. More frequently the removal of a terminal macrozoöid left two or three cells on the last branch (Fig. 1). Simple cut-offs leaving this branch with more than three cells were rarely possible for the reason that a division of the terminal macrozoöid usually preceded the third division on the adjacent branch.

There were a number of cases where, after an operation, the appointed branch continued to develop at a normal rate for one or more generations before another terminal macrozoöid differentiated (Fig. 2). In approximately 12 per cent of the regulating colonies two divisions followed the operation, within normal time limits, before indications of a new terminal macrozoöid appeared. In one case there were three pre-differentiational divisions.

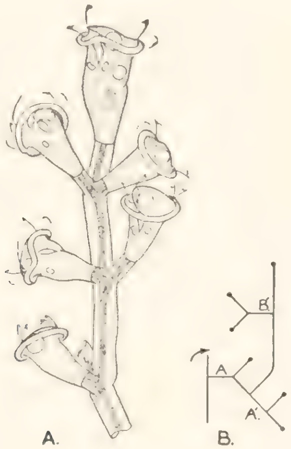

FIG. 2. *A*. Colony 3*II*3-5 drawn approximately 53 hours after the operation. The apical cell was destroyed at the two-cell stage of development. The remaining microzoöid *A* divided twice before the new *T.M.* differentiated from $2a^1$. Its first division produced the initial cell of the branch *B′*. The alternate daughter ($2a^2$) extended the original branch, and the axial microzoöid $1A$ persisted without further change. × 250.

B. Schematic representation of the resulting growth.

Regulation from the terminal branch zoöid $1x$ following simple decapitation of the growing point occurred in a high percentage of the cases (see Tables I and II). As far as the various levels along the primary axis were explored, the cells of the first few branch generations exhibited relatively frequent regulative responses. For the small number of operations made at high levels on old colonies, the transformation of $1x$ into a new terminal macrozoöid occurred with about the same frequency and with as much dispatch as for earlier periods, i.e. lower levels on younger colonies. The metamorphosis of the activated microzoöid $1x$ at levels above E required no more than the average time necessary for differentiation of apical cells produced at lower levels. This appears to correlate with the more or less uniform rate of normal axial growth (Fauré-Fremiet, 1930). Likewise $2x^1$ zoöids on the various branches responded in the majority of trials. Zoöids of the $3x^1$ generation or later failed to regenerate above the mid-region of the experimental colonies.

In order to test the responses of a branch cell of the third generation it was necessary to cut away the newer branches which had formed above it along the main axis. In tests of the fourth or later branch generations, a relatively large part of the colony had to be removed.

Compound Cuts.—When the terminal macrozoöid *and* the terminal cell of the last branch were removed, the terminal cell of the second preceding branch was frequently induced to differentiate into a new terminal macrozoöid. This particular relationship obtained for a limited number of successive branches and even then was unpredictable. For instance, the terminal macrozoöid was sometimes produced by the sub-terminal (a secondary) microzoöid of the newest branch despite the presence of a healthy terminal cell on the penultimate branch. In rare cases the latter assumed the regulative function in the presence of a complete uninjured branch between it and the cut-off.

The number of regenerates obtained from the sub-terminal branch zoöids is given in Table II. Microzoöids of the order $2x^2$ responded up to the level of branch I, whereas sub-terminal zoöids of the fourth generation ($4x^2$) did not respond at all. It is also certain that some colonies did not regenerate from either the sub-terminal zoöid of the first branch below the cut or from the terminal zoöid of the next adjacent branch. These branches continued to develop in a normal fashion for one or more generations without attempting to produce a new terminal macrozoöid.

The reactions of the older segments of well-developed colonies were tested by means of extensive "cut-backs," colonies cut off at some more basal internode. The data obtained (Table II) suggest an inverse relation between the number of regulative responses and the age of the activated zoöids, i.e. the frequency of responses diminishes as the number of lateral generations increases. The frequency of negative cases even in younger generations increased in the high levels, which is probably an expression of the fewer generations required to bring the more distal branches to full development. The same data arranged according to amount of colony removed (Table I) show that basal branches may differentiate a new terminal macrozoöid after as many as seven branches plus the apical zoöid are cut away.

The cut-back experiments were successful only as regards the demonstration of initial reactivity to surgical alterations. The cases in Tables I and II where relatively large portions of the colonies were removed show only that the prospective values of certain zoöids along branch axes may or may not be modified, depending upon the amount of colony dissected away. The capacity of the responding zoöids for sustained growth is not known because all of the colonies cut back four or more branches had to be discarded before the regenerate attained full growth. Indeed, some were maintained under experimental conditions only long enough to produce a new apical cell. The chief difficulty is referable to the fact that the basal branches were the first to be attacked by vegetable growths propagating over the surface of the slide. The affected basal zoöids were shed before the colonies reached maturity. For this reason the zoöids of branch *A* on colonies with eight or more branches were usually unsuitable for testing. The age of the colony at the time of operation plus the additional time required for the differentiation of one of its zoöids gave to the parasites an advantage that was too frequently fatal to the experiment.

Regulation from the Axial Microzoöid Series.—Although nearly 9 per cent of the regenerates sprang from the axial microzoöid (1*X*) series, their regulative behavior was capricious and could not be induced at will. One of the most striking facts in this connection was the origin of new growths from 1*X* or descendants on complete, uninjured branches (Fig. 3). One originated from the third branch below the cut. Deliberate attempts to activate a given axial zoöid by eliminating all other zoöids on the branch resulted in (*a*) no further developmental activity, or (*b*) regeneration from some zoöid on the next lower branch. A three-cell colony which was trimmed down to a single cell, the axial microzoöid 1*A*, remained without further change for 165.5 hours; its contractile and feeding responses appeared to be normal for the entire period of observation. Similarly, the corresponding zoöids on branches *E*, *L*, *M*, and *P* of other colonies were tested without avail. In each case the regenerate developed from zoöids on the next lower branch and is so recorded. On the other hand, it has been demonstrated that the axial microzoöids are capable of regenerating (see 1*X* and $1X^1$ in Table II).

The protocols show that 1*X* or some of its descendants carry terminal macrozoöid potencies. In some of these cases the regenerates formed directly from $1X$ without leaving ciliospore-producing cells on the activated branch. The others regulated after $1X^1$ and $1X^2$ were formed; $1X^1$ gave rise to the new terminal macrozoöid while $1X^2$ produced one or, after division, two typical ciliospores.

Regulation after Successive Cuts.—The successive operations were of two general classes: (*a*) progressive, in which the second and third cuts removed regenerated parts distal to the preceding operation (Fig. 4); and (*b*) regressive, where the entire regenerate plus additional parts of the original colony were cut off. In the first group the second and third regenerates were themselves products of regenerated segments. Those of the second group developed from some more basal

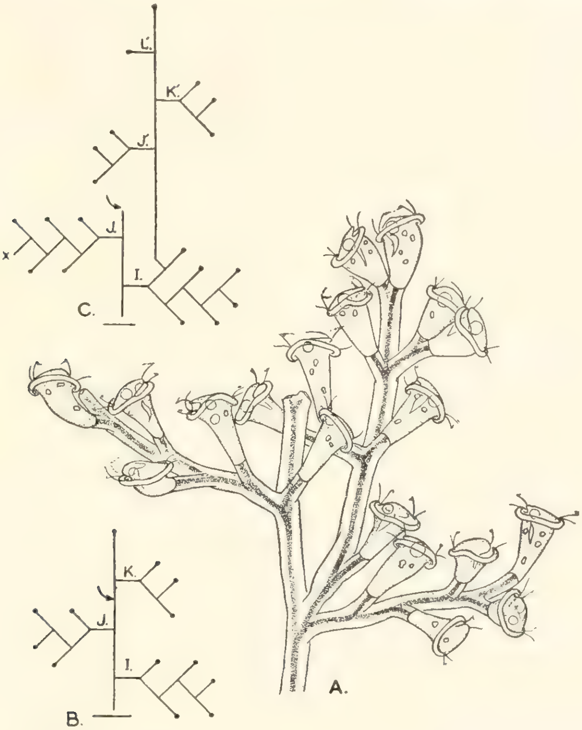

Fig. 3. *A*. Drawing of colony *3IJ-2* approximately 72 hours after cutting. In this case the regenerate was produced by the axial microzoöid on the intact branch *I*. Axial microzoöid $1I$ divided before the new terminal macrozoöid differentiated: $1I^1$ regulated, leaving $1I^2$ near the base of the new axis. An increase in diameter of the new axis is evident near its junction with the original colony axis. × 250.

B. Condition of the apical end of the colony before cutting at the point indicated by the arrow.

C. Schematic representation of the resulting growth at the time the drawing was made.

cell of the original colony. In this respect they were similar to the extensive cut-back types and subject to the same technical limitations.

The regulative activity of the zoöids of the second and third order regenerates in progressively cut colonies was similar in most respects to that evoked after simple cut-offs. In each of the nine cases studied the same morphological pattern and, as far as the limited number of cases permit judgment, similar developmental rates obtained after the second and third operations.

Incomplete Section of the Stalk.—In a few cases out of many trials a local injury to the neuro-muscular cord was effected without destroying the continuity of the cortical hyaline stalk substance. A requisite degree of compression between the needles caused the cord to break down into a series of irregular protoplasmic droplets, some of which appeared to be independent of any attenuated membranous connectives.

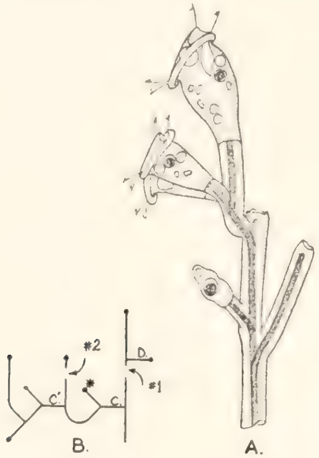

FIG. 4. *A*. Development after two successive operations (54 hours after the first cut). The original axis lies on the right. The severed peduncle of the regenerated terminal macrozoöid may be seen near the base of the second regenerate. Axial microzoöid 1*C* was badly parasitized; it dropped away soon after the drawing was made. × 250.

B. Schematic representation of *A*. The cuts are indicated by arrows. *Parasitized zoöid.

Several interesting facts were brought out by this type of operation. The severed part of the neuro-muscular cord did not recover from the injury, i.e. the structural or functional continuity between the separated parts was not re-established. After the injury there was no subsequent degeneration of the cord in either proximal or distal parts. The functional unity of the whole was permanently impaired. The proximal and distal parts contracted independently of each other and, as established in one case, the distal portion continued to grow and differentiate in the manner of an intact colony, whereas the proximal part regenerated a new primary axis from a zoöid below the injury (Fig. 5).

More substantial data are obviously required before definite conclusions can be drawn. Nevertheless these results do suggest interesting possibilities for further study. A thoroughgoing investigation of the contractile, transportative, and transmissive properties of the neuro-muscular cord may lead to a further elucidation of coördinating factors in colony formation. At least here is a clear indication that, whatever the physico-chemical nature of the integrative factors, they are probably mediated through the substance of the cord.

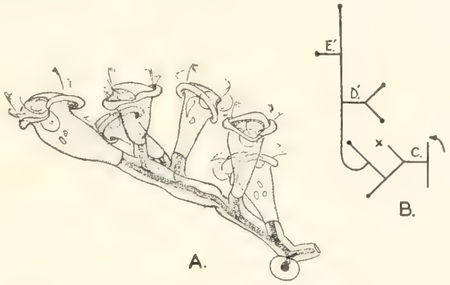

FIG. 5. *A*. Branch *C* of colony 9*B*4 56 hours after injury to the neuro-muscular cord (drawn from above). The original colony of six branches was pinched in the mid-region, isolating *ABC* from *DEF* plus the terminal macrozoöid. The terminal cell on branch *C* ($3c^1$) at the time of the operation differentiated into a new terminal macrozoöid which produced two new branches as shown. Note the increased diameter of the stalk just lateral to the second zoöid. This marks the position of the microzoöid $3c^1$ at the time of injury. × 250.

B. Schematic representation of branch *C* as drawn. Axial microzoöid 1*C* was accidentally cut away from the position marked (*x*).

Several completely isolated fragments were followed for a time by transferring every few hours to fresh filtered sea water. Nothing of unusual interest occurred in their development. Growth, differentiation, and regulation in progress at the time of cutting continued as before for the few generations that were followed. They did not re-attach to the substrate but developed as free-swimming fragments. Their growth capacities or the minimum size necessary for survival were not investigated.

Differentiation in Regenerated Parts.—The regenerates formed on decapitated colonies, after single or successive operations, are capable of producing any of the six types of heteromorphic zoöids previously described (Summers, 1938). According to the data compiled from

77 protocols the type, number, and distribution of zoöids on the regenerated parts compare favorably with the control colonies. The regenerates consisted of a new terminal macrozoöid, varying numbers of common microzoöids, a terminal cell at the tip of each branch, and one or more potential ciliospores, depending upon the degree of development following an operation. Macro- and microgamonts likewise differentiated on regulating parts with about the same frequency and vertical distribution as for corresponding regions of normal gamont-producing colonies. Regenerates sometimes differed from the controls in respect to the branch generation involved in the production of ciliospores and microgamonts. In normal colonies the ciliospores developed not earlier than the 1*X* generation and the microgamonts only from $2x^1$ or succeeding generations. On the regenerates one or the other of these two types of migrating zoöids frequently developed from the initial branch zoöid (Fig. 6*B*). This tendency towards earlier differentiation obtained not only in the young regenerates on immature colonies but also in those derived from older (basal) zoöids of nearly mature colonies.

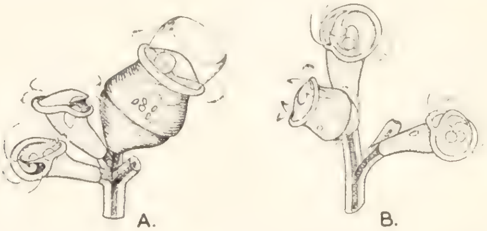

FIG. 6. *A*. Terminal macrozoöid 13′ (above branch *M*) differentiating into a ciliospore 204 hours after cutting. The colony was sectioned between branches *E* and *F*. × 250.

B. Metamorphosis of the median microzoöid *S*′ into a microgamont. The drawing was made 127 hours after regulation from the axial microzoöid 1*O*. × 250.

Another noticeable deviation from the established norm was the occasional metamorphosis of the terminal macrozoöid into a migratory zoöid, either ciliospore or microgamont (Fig. 6*A*), thus bringing to a close the growth along that particular axis. The terminal macrozoöid of the regenerate may differentiate directly into any of the three reproductive zoöids: microgamont, macrogamont, or ciliospore. Fauré-Fremiet (1930) reported the formation of the latter type in rare instances during the normal development of this species but the process was unaccompanied by the endomictic process which he described for normal ciliospore development.

While studying conjugation in *Zoöthamnium arbuscula*, Furssenko

(1929) observed that a local injury to one of the main branches affected the zoöids distal to the region of injury, inducing many of the microzoöids to metamorphose into microgamonts ("microconjugants"). Similar effects on the whole colony were induced by unfavorable environmental conditions, e.g. inanition or lack of oxygen. He did not observe a regulatory response subsequent to the injury.

Regulation after Conjugation.—From the following fragmentary account of the growth activity manifest in conjugating colonies it is at once clear that this aspect of development alone constitutes a lengthy research problem. Only ten of the colonies whose lineage was being followed happened to conjugate so that a detailed analysis of the process is not immediately available. Much of that which follows is based upon conjugants observed among the adventitious growths on the culture slides whose histories are but imperfectly known. Conjugation is introduced here because it affords one clue to qualitatively different physiological relations between the apical zoöid, the conjugant in this case, and a large area of the subordinate regions of the colony.

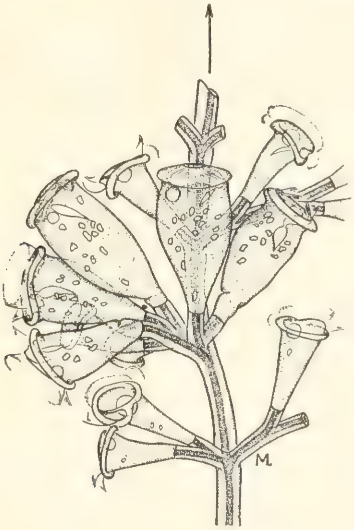

FIG. 7. A colony several days after the onset of conjugation at the level of branch *N*. The ex-conjugant divided into a cluster of large zoöids, one of which differentiated into a new *T.M.* whose further development prolonged the main axis. The exact lineage of these cells is not known. × 250.

So far the results obtained from regulation experiments bespeak a regular functional correlation between the single cell in the apical position and the zoöids in sub-adjacent regions, such that the latter are subservient to the former. Potentialities known to be present in zoöids of a lower order are presumably held in abeyance by the apical influence. Cutting away the apical region evokes a response in some zoöid in a subordinate but adjacent region. The response is a differentiation of another apical zoöid whose relations with the whole are seemingly homologous with those of the original apical cell. There may be a time between decapitation and subsequent regulation when the apical cell influences are altogether absent, yet the interim is not sufficiently great to seriously modify the observable growth phenomena. It is noteworthy that subordinate branches attain about the same end-point of lateral growth in control and decapitated colonies.

As stated in the previous work (Summers, 1938), the macrogamonts were observed only in the terminal macrozoöid positions 3 to 24 along the primary axis. The fusion of gamonts invariably brought axial development temporarily to a close some 12 to 13 hours after the last mitosis.

The conjugant remained quiescent for periods of about four days, then divided into two moderately large zoöids. One of the two ex-conjugants assumed the form of a terminal macrozoöid and resumed axial development after the four-day interruption. The fate of the sister ex-conjugant is a matter for conjecture at present; some disappeared from the colonies between observational periods while others divided into clusters of from two to seven large ciliospore-like zoöids at the base of the new axis (Fig. 7). The histories of these are likewise unknown. Apparently they do not propagate additional axes while associated with the parent colony. The development of ciliospores from some of the ex-conjugants in *Zoöthamnium arbuscula* (Furssenko, 1929) is suggestive, however.

The point to be made relates primarily to the behavior of the colony as a whole following the conjugation process. Prior to the completion of conjugation and continuing thereafter a new growth phenomenon appeared. The first three or four branches below the presiding conjugant developed out of all proportion to the average expectations (Fig. 8). The number of branch generations was in some instances greater than twice that of corresponding branches in controls. Moreover, many of the common lateral or secondary zoöids were activated to divide one or more times, originating second order branches which, in turn, sometimes produced tertiary branches. In this way each of the first few branches below the conjugant level grew almost as individual colonies. The greatest lateral growth effect obtained nearest the conjugant and diminished basally as a gradient. The normal tendency toward a pyramidal colony pattern was thereby reversed in the environs of the conjugant. More precise information regarding growth intensity and capacity factors in both the ex-conjugant strain and the subordinate branches awaits further investigation.

Discussion

One of the most important consequences of the work is the demonstration of qualitatively different physiological relations between spatially separated cells. Under normal conditions a specific pattern unfolds. When an apical region of a colony is cut away some zoöid of a lower order, one whose complete developmental possibilities are otherwise never expressed, assumes the dominant generative functions, and the characteristic pattern perseveres. So far these results are intelligible in terms of what Child (1929) calls physiological correlation: the relations of dominance or control and subordination between

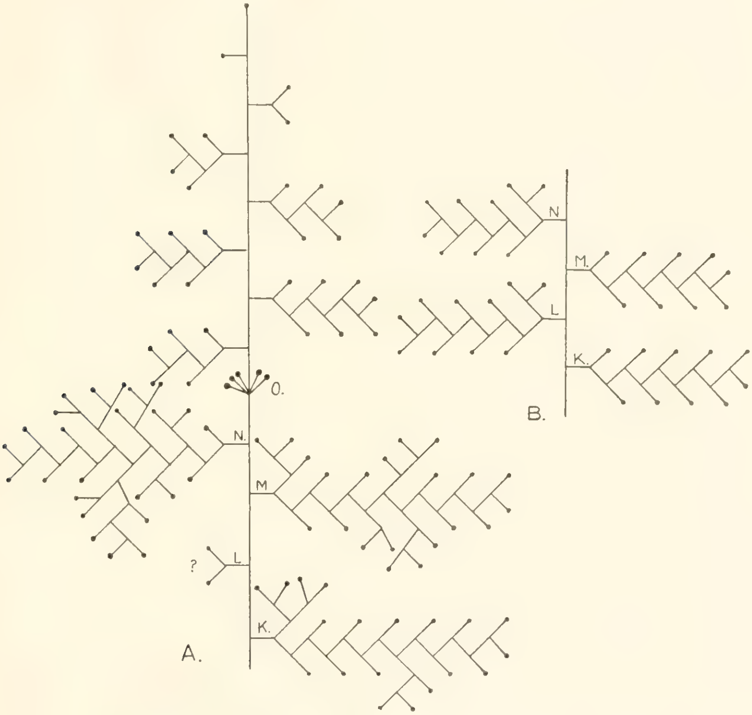

Fig. 8. *A*. Schematic representation of protocol 19*oe* to show the disproportionate development of branches *K*, *M*, and *N*. The lineage of branch *L* could not be deciphered. One of the ex-conjugant zoöids at *O* produced the apical growth illustrated in the diagram.

B. The corresponding portion of the largest of the 70 control colonies.

parts. He concludes "that dominance and subordination depend primarily on quantitative, rather than specific differences in physiological condition and that they represent a certain aspect of a physiological gradient." In *Zoöthamnium alternans* the transformation of a terminal macrozoöid into an ex-conjugant initiates an entirely different developmental phase which gives another clue to the general nature of apical control. Four days after the fusion of gamonts the normal growth relations of a varying number of branches below the conjugant level are upset in a rather remarkable way. Each of the three or four adjacent branches develops out of all proportion to the normal expectations. The precocious mitotic activity produces secondary and even tertiary axes on the affected parts. This unusual phenomenon does not occur when the terminal macrozoöid is present *or* when it is absent; it is effected by some new quality in the coördinating mechanism arising in consequence of conjugation activities in one particular cell—the apical cell.

The effects obtained after decapitation and conjugation certainly suggest that the single cell in the apical position is responsible in a large measure for quantitative and qualitative regulation of the part-whole relationship and that the control varies with respect to the local activities of the parts.

There also seems to be a coördination between the cells on different branches. A new terminal macrozoöid arising from one of the branch cells exerts its influence from what was formerly considered to be a branch position. Perhaps branch-to-branch coördination also explains the stable activity of the variously placed cells in the interim before a new apical cell differentiates; only one of the several possible zoöids regulates. The control of a terminal branch cell over the cells on its own branch can be interpreted in a similar way. As long as the terminal cell presides over a branch strain its immediate relatives remain quiescent. If it is destroyed, however, the sister at the subterminal position assumes the functional rôle of the lost cell.

A rather wide variation in the degree of regional correlation is suggested by those instances where a new terminal macrozoöid arises, not on the first branch as usual, but on the second or even third branch below the operated level.

The axial microzoöids on every branch do not differentiate into ciliospores. Loci of metamorphosing zoöids occur on about every third or fourth branch. The prospective value of a single axial microzoöid ($1X$) is predictable at a relatively early period by a marked growth in size. The growth may be taken as a criterion of at least a partial differentiation, to be completed a good many hours later by a further increment in volume, modification of form, appearance of motile organelles, etc. A peculiar characteristic of these large cells is that they may differentiate directly into mature ciliospores or they may divide, giving origin to two zoöids of unequal size, both larger than any of the common types. The larger of the two matures first; the smaller grows to the size of its predecessor before metamorphosing or dividing again to produce two mature ciliospores in succession.

Regenerates sometimes arise from either those axial microzoöids which are, to all appearances, not predestined to metamorphose, or from those already partly differentiated. In the latter a potency or potencies in the process of expression apparently can be altered or superseded by others whose "urgency" toward expression is greater. The directional change in the process is referable to stimuli arising from the altered colonial organization. This is but another bit of evidence to the effect that cellular organization is dynamic and labile at certain periods and that changes going on within the cell which lead to recognizable morphogenetic characteristics are not necessarily irreversibly determined in direction. Many cases are known among the Protozoa where extrinsic or intrinsic factors lead to periods of reorganization, varying in considerable degree for the different groups and at different periods in the life cycle. In *Zoöthamnium alternans* we have a case where the re-direction of morphogenetic processes can be traced to an extrinsic cause: cutting the colony in the near vicinity of the cell subsequently affected.

Several significant problems arise in connection with the variable response of the axial microzoöids. Why do regenerates sometimes arise from axial microzoöids (1*X* or descendants) when as a rule the new growths are derived from the terminal branch cells? Attempts to induce regulation from 1*X* cells by trimming away all other cells on the branch gave no positive or predictable results. Until the question is investigated further in *Zoöthamnium* we can only interpret the variable behavior in terms of other work. Some of the merotomy experiments on other protozoa are suggestive (Calkins, 1911*a*, *b;* Peebles, 1912; Young, 1922; Dembowska, 1926; Taylor, 1928; and others). With respect to regenerative capacity these investigators were able to demonstrate progressive physiological changes in the cellular organization during the inter-mitotic period. Fragments cut at successive intervals after fission gave an increasingly high percentage of perfect regenerates. In *Uronychia* (Calkins, 1911*a*) even an emicronucleated fragment regenerated when cut immediately before the onset of fission. But in nearly all of the different forms studied the regenerative tendency disappeared sometime during the division process.

Another line of investigation summarized by Calkins (1934) and Summers (1935) demonstrates the cytological changes in cell organelles coincident with the division process. The resorption of old and the reappearance of new motor organelles, macronuclear reorganization, etc. suggest a brief period of cellular de-differentiation. There are probably analogous processes of alternating differentiation and de-differentiation in the history of the individual zoöids in *Zoöthamnium*. Time may be one of the important factors in individual cell behavior in relation to the balance between extrinsic coördinating influences and the aggregate of intracellular activities. That is to say, the intracellular activities of a *Zoöthamnium* cell may lead to the "fixation" of specific potencies at some critical period after cell division or, conversely, a cell may be more susceptible to the coördinating influences during or immediately after a division process. The axial microzoöid, for example, divides in from 20 to 70 hours after its derivation from the initial branch cell, whereas the terminal cell on the branch generally divides at intervals of about 12 hours. If a decapitation is made at a moment when the axial microzoöid is in some phase of divisional reorganization and the terminal cell in a more stable condition, the former instead of the latter may be activated or excited to prolonged generative activity. The supposition should be tested by a series of accurately timed operations above some particular branch.

An explanation of morphogenetic processes in *Zoöthamnium alternans* in terms of embryonic segregation at the time of division has already been attempted by Fauré-Fremiet (1930). In order to outline several points for discussion it is essential to review briefly his cytological analysis of normal development in this species. First, as regards the early axial divisions, the first three generations along the primary axis are unequal divisions. The inequality of the resulting daughters is reflected in the assortment of macronuclear material; each time the zoöids which remain in the terminal position (*TM*. 1, 2, 3) receive a larger portion of the macronuclei than the smaller branch microzoöids (*A*, *B*, *C*). On the supposition that the enlarged end of the macronucleus apportioned to the terminal macrozoöids represents a kind of segregation of chromatin material, these three unequal divisions are described by Fauré-Fremiet as qualitatively and quantitatively differential divisions. Beginning with the fourth division (division of *TM*. 3) the extremities of the dividing macronuclei in all later axial divisions are similar in size but in each instance a bit more of the finely striated mid-portion of the macronucleus is received by the zoöid remaining in the terminal position. All of these later divisions are characterized as quantitatively differential only. With respect to the branch generations, all divisions along branches *A*, *B*, and *C* are similar and almost equal. The initial zoöids of subsequently produced branches (*D*, *E*, *F*, etc.) undergo qualitatively differential divisions: the axial microzoöids (1*D*, 1*E*, 1*F*, etc.) receive a greater share of the macronuclei than their lateral sisters (1*d*, 1*e*, 1*f*, etc.) although the cytoplasm in each case is distributed equally. The axial microzoöids (1*D*, 1*E*, 1*F*, etc.) represent the ciliospore-producing members of the colony. They undergo marked growth in size accompanied by a disintegration and reconstitution of the macronuclei which, although not described in detail, is characterized as an endomictic process. Fission in the lateral sisters (1*d*, 1*e*, 1*f*, etc.) is of no further interest cytologically; these zoöids constitute the main branch strains.

From the foregoing description it follows that differential division occurs at two points in the formation of all branches above *C*. To illustrate (Fig. 9): *D* receives less cytoplasm and macronucleus than *TM*. 4, but the macronucleus in both resulting zoöids is qualitatively similar. The division is therefore quantitatively differential. When the initial branch cell *D* divides its cytoplasm is distributed equally but the macronucleus is assorted differentially because a thickened granular part goes wholly to the axial microzoöid 1*D*. The division is therefore qualitatively differential.

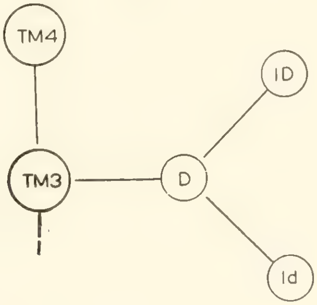

FIG. 9.

According to Fauré-Fremiet, "It appears clearly then that during the growth of a colony of *Zoöthamnium alternans* the two cells resulting from a division of one initial cell are never equivalent as to their 'potentialities.'" Also, "The character of the differential divisions on the main strain seems to determine the individual's differentiation of the colony; this differentiation depends not only on the individual's size, but also upon its physiological potencies." The differential divisions also appear to determine the characteristic features of the median individuals and of the microzoöids. The latter have a limited power of growth and multiplication. Median microzoöids on branches *A*, *B*, and *C* remain as common nutritive zoöids, whereas the corresponding zoöids or their descendants on branches above *C* may undergo considerable growth and metamorphose into ciliospores.

Is it to be inferred from this analysis that quantitatively differential divisions determine (restrict) the subsequent power of division in branch strains and, further, that qualitatively differential divisions effect a segregation of potencies for ciliospore formation?

Regarding the first inference, all alternating median microzoöids, initial branch cells, are the lesser products of quantitatively differential divisions; they can divide only so many times, according to the number of generations normally produced on the branches of which they represent the beginning. While there is no doubt about the inequality of the cytoplasmic distribution between terminal macrozoöids and initial branch cells for the first ten generations or more, the inequality diminishes beyond this point until the two daughters are no longer differential as regards volume of cytoplasm. An equality may be achieved as early as the tenth and not later than the twentieth axial generation. Then what of those colonies that developed eight to fifteen generations beyond the twentieth node with similar axial-lateral relations? Another point to be made pertains to the regulative capacity of the branch zoöids. Those distributed in alternate positions along a branch axis seldom divide further so long as the integrity of the whole colony is preserved. When an apical piece is cut away from the colony some one of the more lateral zoöids on the remaining portion is capable of assuming the principal generative functions for relatively long periods of time. This behavior is difficult to interpret on the assumption that mitotic or "growth" potentialities are conditioned at either or both of the first two generations on the regulating branch.

The second inference may be challenged upon the grounds that segregation of ciliospore potencies does not occur at the specified division. Fauré-Fremiet adduces cytological evidence of segregation at the first three axial divisions and thereafter at the division of the initial branch zoöids. The latter is the fission at which the ciliospore-forming zoöids are separated from the main branch strain. In the light of newer findings, the restriction of ciliospore formation to 1*D*, 1*E*, 1*F*, etc. can be questioned. Ciliospores were observed to develop from axial microzoöids on the second and third branches, and also from both daughters of the fifth and tenth generations on branches *D* and *H* respectively of control colonies. Moreover, ciliospores occurred on the regenerate in nearly every case of regulation from branch zoöids lateral to the supposed differential division provided, of course, that they were maintained for a sufficient length of time. The final bit of evidence comes from the demonstrated regulative capacity of the axial microzoöids ($1X$) on some of the operated colonies. When activated these were able to regenerate comparatively large sections of colony upon which all classes of zoöids except gamonts appeared.

The spatial relationship of cells should not be minimized as an important determining factor in an organism whose cells are so characteristically placed. The importance of this factor in development cannot be properly valuated until the general physico-chemical nature of the integrating mechanism and the medium through which it operates, presumably the neuro-muscular cord, are more fully understood. It is fairly certain, however, that it is not a specific factor, for in normal uncut colonies ciliospores occasionally develop in odd positions, and the microgamonts are apt to differentiate from the common zoöids at almost any position lateral to the axial microzoöids. Of related interest is the work of Buchanan (1927) on the flatworm *Phagocata*. The region from which a piece is taken with reference to the mouth of the intact worm is of no significance in determining the location of the mouth in the regenerate. Seyd (1935), on the other hand, reported a definite degree of regional specificity in the regeneration of a new mouth in *Spirostomum;* mouths in abnormal positions in the cut organisms degenerated and new ones formed at the correct locations.

The conjugation processes in *Zoöthamnium arbuscula* (Furssenko, 1929) and *Z. alternans* do not differ in essential detail. In the former the zoöids at the terminal position on each of the two primary axes (A_e and B_c) becomes the "macroconjugants." Until metagamic divisions occur, further growth along these axes is arrested. Two metagamic divisions of the conjugant result in a cluster of four large zoöids, two of which (A_2 and C_2) metamorphose directly into macrozoöids (ciliospores). The remaining two (B_2 and D_1) divide again, each giving rise to another macrozoöid and a stem cell. A stem cell produces an additional macrozoöid and a new (ex-conjugant) axis. A single conjugant therefore produces two ex-conjugant axes and from four to six successively produced macrozoöids.

The behavior of small lateral branches from the main axis at nodes basal to the conjugant in *Z. arbuscula* compares favorably with the precocious development of subordinate branches on a conjugating colony of *Z. alternans*. One or two of the small lateral branches (Seitenätschen) below the conjugant develop to the proportions of regular main branches. In this fashion normal colonies with seven main axes are transformed, after conjugation, into colonies with nine to eleven chief axes. Furssenko refers to the new growths as "compensation" branches (Ersatzzweigen). It is his belief that the enormous growth of the macrozoöids occurs at the expense of food obtained from nearby microzoöids and, similarly, the compensation branches are destined to supply the energy needs of the ex-conjugant derivatives, i.e. the five or six clustered macrozoöids and the new ex-conjugant axes.

The chief support of Furssenko's hypothesis that ex-conjugant generations develop at the expense of adjacent regions comes from two observations: (*a*) the ex-conjugants themselves are not active feeders, and (*b*) the macrozoöids in neighboring regions either fail to mature or they divide to form common zoöids. The idea presupposes a mobilization and free transport of nourishment to regions active in development.

The observations on *Zoöthamnium alternans* are not in any way contrary to a possible rôle of the stalk in transportative phenomena. It is also quite likely, although yet to be proved, that nutrient materials are utilized by some zoöids at the expense of adjacent ones; this may be a cause contributing to growth inhibition or differentiation in nearby cells. Nevertheless it does appear that the precocious development of subordinate branches in a conjugating colony of *Z. alternans* is not primarily directed toward nutritional ends. In the first place, the actively developing apices of branch strains have energy requirements which, when taken as a whole, undoubtedly exceed the demands of the single ex-conjugant or its first few non-feeding descendants. The flux would therefore be directed away from the conjugant node. Secondly, in nearly every instance recorded the unusual development of the subordinate branches was well under way before the first post-conjugant division occurred. It is problematical whether or not the change in food requirements coincident with the transformation of a terminal macrozoöid into an ex-conjugant is sufficiently great to account for the relatively far-reaching alteration of the growth pattern.

SUMMARY

1. *Zoöthamnium alternans* is a colonial protozoan of a rather special type whose constituent cells collectively possess in some degree many of the attributes of an integrated organism. Some of the integrating factors can be described in general terms from the work undertaken on form regulation.

2. When the apical cell of the primary axis is dissected away from a developing colony,, a cell on some inferior branch, usually the first

below the cut, will differentiate into a new apical cell. The geographical limits within which positive regulative responses occur are given in the text.

3. Development of a colony continues from the newly differentiated apical cell. The structural and developmental characteristics of the normal colony persevere in the regenerated portion.

4. Evidence is presented to the effect that zoöids retain, for a time at least, greater developmental potentialities than are actually expressed when they comprise a part of the intact colony.

5. Under varying physiological conditions in the apical region of a colony, the coördinating influences exerted upon the mitotic activity of neighboring zoöids may be inhibitory (as shown by the responses evoked after decapitation) or excitatory (when the terminal macrozoöid is transformed into an ex-conjugant).

6. In the light of observations presented, the idea of dichotomous segregation or sifting out of potencies at fission is inadequate as an explanation of localization in this species. The experimental data do not confirm Fauré-Fremiet's cytological account of qualitatively differential divisions at specified division nodes on the branches.

7. There is cause to suspect that morphogenetic processes in particular zoöids of *Zoöthamnium alternans* (e.g. the presumptive ciliospores), once initiated and partly expressed in visible structure, can be conditioned or modified by cuts made in some neighboring region.

8. An hypothesis is offered to account for the origin of a regenerate from one or the other of several dissimilar cells of a branch strain. The explanation is based upon the factor of time in relation to the balance between extrinsic influences and the aggregate of intracellular metabolic activities by which potentialities are realized. The cells are thought to be more susceptible to external control during the reorganizational period of mitosis. There may be a critical time in cellular differentiation beyond which the intrinsic processes are not influenced by stimuli arising in some other part of the colony.

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QUANTITATIVE STUDIES OF THE FACTORS GOVERNING THE RATE OF REGENERATION IN TUBULARIA

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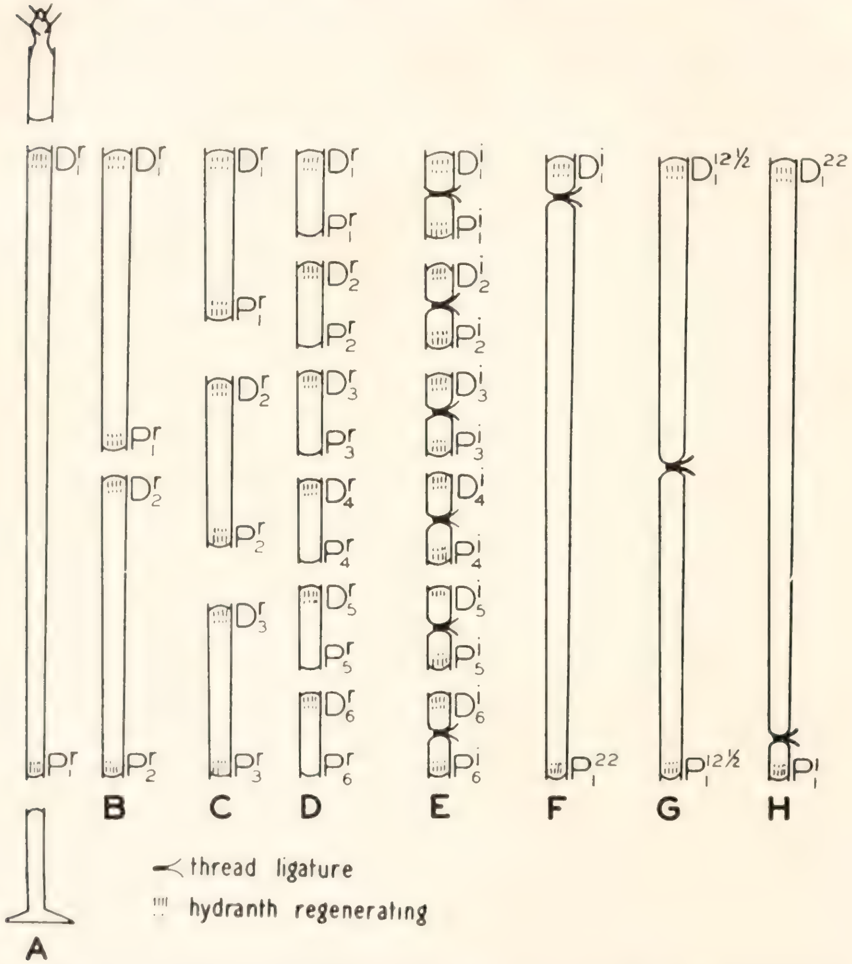
The purpose of these investigations is to determine the reciprocal effect of two regenerating regions in *Tubularia*. In order properly to design the experiment it is necessary first to work out the rates of regeneration of different parts of the stem and to determine the effect of size of stem on rate of regeneration. Following this two regions of known rates are allowed to compete with one another and the effect on rate of regeneration is measured. Since it is found that the region exhibiting the lower rate is most affected, further experiments are designed to measure this effect. This inhibitory effect exercised by the higher rate over the lower rate has been termed physiological dominance by Child (1929) and the term "dominance" is used in this paper to describe inhibition.

When the stem of *Tubularia* is sectioned the hydranth differentiates *in situ* from the tissue of the cut end and after 30–40 hours at 18° C. the length of the primordium can be measured by means of an ocular micrometer. At this time a constriction appears at the base of the developing hydranth separating it from the rest of the stem. The perisarc being rigid, the diameter of the hydranth can be measured and the volume calculated. The time for regeneration is measured at two stages of development. t_1 is the time in hours from the time at which the stem is cut to the time at which the constriction appears between the primordium and the rest of the stem. t_2 is the time for complete regeneration when the hydranth is pushed out of the opening of the perisarc. In some experiments where short pieces of stem are used it is necessary to use t_1 as a measure of time since the hydranth, although completely differentiated, does not emerge from the end. In most cases, however, t_2 is used for the calculation of rate of regeneration.

Rate of regeneration can then be defined as the volume of the hydranth in cubic micra divided by the time in hours. $R = \pi r^2 L/t$.

Naturally when t_1 is used the rate is somewhat higher than when t_2 is the measure of time. The values obtained are in regeneration units which are termed R. U. in this paper.

It is convenient to assign symbols to the ends and this has been done in Fig. 1 which shows the method of naming the hydranths. D_1, D_2, D_3, etc. are used to designate the distal or oral hydranth of each piece of the stem and are numbered consecutively from distal to



D_1^r
P_1^r
D_2^r
P_2^r
D_3^r
P_3^r
D_4^r
P_4^r
D_5^r
P_5^r
D_6^r
P_6^r
D_1^i
P_1^i
D_2^i
P_2^i
D_3^i
P_3^i
D_4^i
P_4^i
D_5^i
P_5^i
D_6^i
P_6^i
P_1^{22}
$D_1^{12½}$
$P_1^{12½}$
D_1^{22}
A
B
C
D
E
F
G
H
thread ligature
hydranth regenerating

FIG. 1. *A* shows how the stems are prepared; *B*, *C*, *D* how halves, thirds and sixths are prepared and designated; *E* shows how inherent rates are determined by tying off a small part of the stem; finally, *F*, *G*, *H* show how the length of stem is varied while the level remains constant. D_1^r, D_2^r ⋯ relative rate of distal hydranths; P_1^r, P_2^r ⋯ relative rate of proximal hydranths; D_1^i, D_2^i ⋯ inherent rate of distal hydranths; P_1^i, P_2^i ⋯ inherent rate of proximal hydranths. D^{22} and P^{22} are examples of absolute rates.

proximal levels. D_1 is always the most distal hydranth of the stem. P_1, P_2, P_3, etc. are used for the proximal or aboral hydranths in the same order. A glance at Fig. 1, *A*, *B*, *C*, will make this clear.

The stems are kept in a rectangular dish which is cooled by a glass coil through which running sea water is circulated. The stems rest on a strip of cheesecloth which is stretched over a glass frame and are covered by one centimeter of sea water. Loose-fitting glass covers are placed over the top of the dish. A glass rod, which is drawn out and bent in the form of a hook, is used for picking up and transferring long stems. For short stems a pipette bent at a 45° angle is satisfactory.

Since the stems of *Tubularia* are very variable in the rate at which they regenerate, controls were prepared for every experiment, and experiments involving the use of different stems are never compared. That is to say, one may not compare the rates of 15-mm. stems from different experiments for they may be vastly different, as measurements show (Table II, Experiments 5, 6 and 7).

The general procedure was to select from several colonies those colonies with straight stems and no side branches. The stems were cut off at the base and placed in a large dish of sea water and further selected for similarity in length, diameter and appearance. Some stems were translucent, while others were opaque, and these two differed in rate of regeneration. After this selection, the hydranths were cut off, and the stems of the same length were selected at random for experimentals and controls. In long stems, the additional precaution of cutting off 3 mm. of the stem along with the hydranth was observed, as the region adjacent to the hydranth often regenerates at a low rate, due possibly to the use of this region in the formation of the very large gonophores.

The Inherent Rates of Regeneration at Various Regions of the Stem

The object of these experiments is to isolate various levels of the stem so that the inherent rate of regeneration can be determined. Isolation of a region from the rest of the stem can be obtained by means of a thread ligature which is tied about the stem shutting off circulation and cutting through the tissue. This technique was used by Morgan (1902) and lately by Peebles (1931). The perisarc, which is tough, does not crack but the coenosarc is completely severed so that there are no cellular connections across the ligature. If this ligature be applied about 2–3 mm. from the end of the stem a small piece of tissue 2–3 mm. in length is isolated and will form a hydranth. By this means the rate of regeneration of a small piece of tissue at any level of the stem can be measured without being influenced by the stem as a whole. The situation is analogous to self-differentiation of an explant from an embryo. It might be thought that the same result could be brought about more simply by cutting off a piece 2–3 mm. in length. However, in this case one meets with the difficulty that two centers of regeneration arise at the two cut ends and bipolar forms may arise. It is impossible to get any measure of the rate at which these forms develop.

TABLE I

Inherent rates of regeneration of distal and proximal hydranths at various levels of the stem of *Tubularia.* Stems 30 mm. long, cut into 6 pieces and each piece ligatured in middle. L = length in μ, d = diameter of stem in μ, t_1 = time in hours from cutting to the constriction of the regenerating hydranth.

	L	d	t_1	$\frac{L}{t_1}$	$\frac{\pi r^2 L}{t_1} \cdot 10^{5}$
D_1	1136	504	31.6	36.0	71.6
D_2	1044	488	34.4	30.3	56.4
D_3	928	456	36.4	26.2	43.3
D_4	880	432	39.6	22.2	33.1
D_5	592	388	39.8	14.8	17.6
D_6	404	364	49.1	8.2	8.7
P_1	924	504	39.1	23.6	47.0
P_2	824	456	39.3	21.0	33.9
P_3	844	432	40.9	20.6	30.3
P_4	704	408	47.5	14.8	19.4
P_5	416	372	44.5	9.2	9.6
P_6	244	340	52.8	4.6	4.2

The method of determining the inherent rates of regeneration at various levels of the stem is shown in Fig. 1, *E,* where the superscript "*i*" is used to designate inherent rate. Stems 30 mm. in length are first cut into 6 pieces and then each 5-mm. piece is ligatured in the middle. Thus in each 5-mm. stem the distal half is completely isolated from the proximal half. Since the stem which regenerates is so short, 2.5 mm., the hydranths have difficulty in emerging and so the time recorded is t_1. This is the time from cutting to the formation of the primordium of the hydranth.

In Table I and Fig. 2 the data are recorded and plotted. The rates are calculated in two ways. $R_1 = \frac{L}{t_1}$ and also $R_2 = \pi r^2 L/t_1$ where L = length of primordium in micra, r = radius of stem in micra and t_1 = time in hours. From the table it is seen that there is a gradient in the size of the hydranth and the time for regeneration. The rate of regeneration R_2 of the distal hydranths $D_1 - D_6$ thus falls off sharply from $D_1^i = 71.6$ R. U. at the distal end to $D_6^i = 8.7$ R. U. at 5 mm. The lowest rate at 0 mm. is $P_6^i = 4.2$ R. U. Thus the most distal level of the stem regenerates at almost 18 times the rate of the most proximal level. This difference in rate must be due to causes inherent in the tissue at these levels since external influences have been removed.

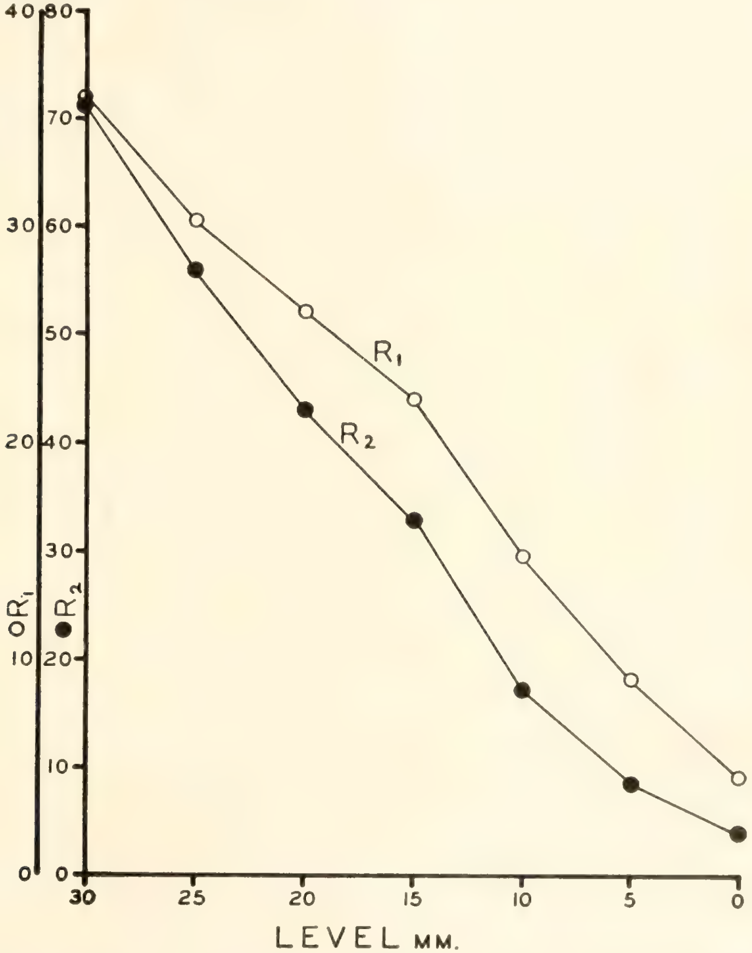


FIG. 2. Rates of regeneration at different levels of the stem of *Tubularia*. $R_1 = \frac{L}{t_1}$; $R_2 = \pi r^2 L/t_1 = \mu^3 \cdot 10^5/\text{hours}$. Stems 30 mm. in length cut into 6 pieces and ligatured in middle. Each point is the average of 10 stems. r = radius of cross-section of stem; L = length of primordium; t_1 = time in hours between cutting and the formation of the primordium. The lowest rate is rate of P_6. Zero mm. is at the proximal end of the stem, 30 mm. at the distal end.

The rate of regeneration R_1 has also been calculated using length as a measure of the size of the primordium in place of volume. These calculations are included here to show that the difference in rate of the regenerating hydranths at various levels of the stem is not due merely to a difference in the diameter of the stem but also to a difference in the length of the primordium. What R_1 really represents is the rate of regeneration R_2 divided by the cross-sectional area of the stem and therefore it corrects for the variation in diameter caused by the growth form of the stem. For obviously if the stem is always wider at the distal end as compared with the proximal end, the apparent rate of

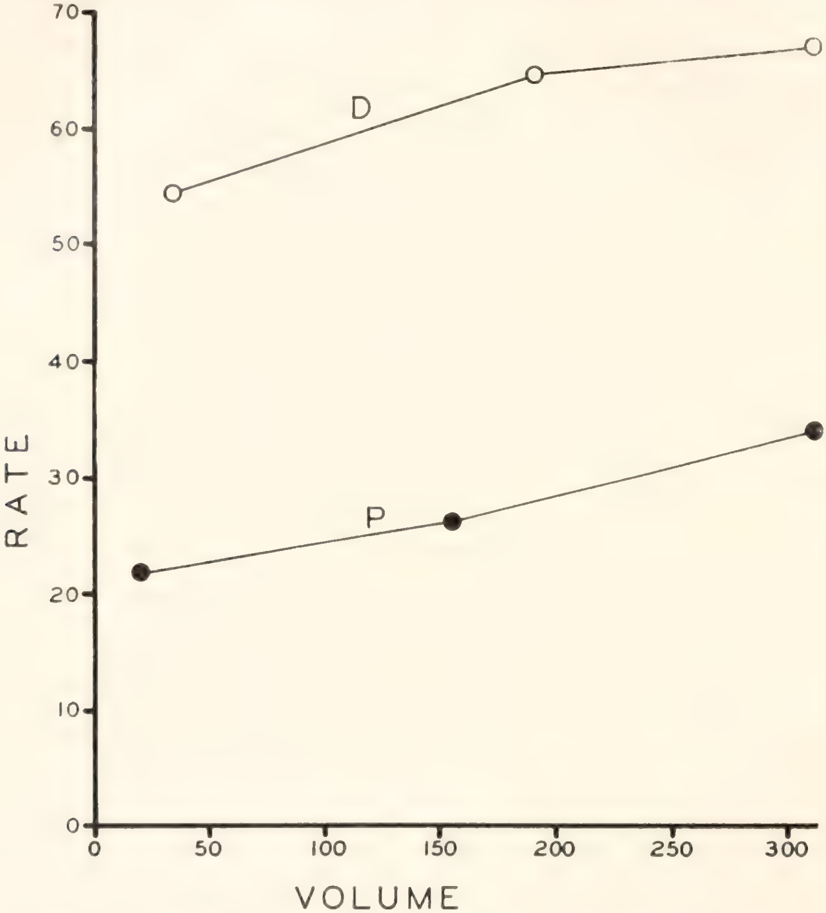


FIG. 3. The effect of volume of stem upon rate of regeneration of hydranth of *Tubularia*. D = distal hydranth; P = proximal. Rate = $\mu^3/\text{hrs.} \cdot 10^5$. Volume = $\mu^3 \cdot 10^7$. Time in this experiment is t_2.

regeneration using volume of stem as a measure of size would always be greater at the distal end even if the time for regeneration and the length of the primordium were the same as those of the proximal end. Such a difference in rate seems to me to be entirely due to morphological reasons and would not represent at all the inherent regenerative activity of the tissues themselves.

From the results (Table I, Fig. 2), however, it is clear that the difference in rate at various levels is a real one and that both the time for regeneration and the length of the regenerate vary in different regions of the stem. When R_1 is used as a measure of rate, the difference in distal and proximal levels of the stem is not so great as R_2 shows. The rate of regeneration of the most distal hydranths $D_1{}^i$ is 36.0 R. U. while that of the most proximal hydranth $P_6{}^i$ is 4.6 R. U., or about an 8-fold difference.

Table II

Relative rates of regeneration in *Tubularia*. Both hydranths are allowed to regenerate. D^r = relative rate of distal hydranth, P^r = relative rate of proximal hydranth. Rate = μ^3/hours$\cdot 10^5$.

Experiment	Length	No.	D^r	P^r	Thickness in μ		Vol. Stem
					D	P	
	mm.						$\mu^3 \cdot 10^7$
1	5	6	68.5	0	—	—	—
2	5	21	60.5	0	—	—	—
3	6	25	95.0	4.65	568	528	141
4	10	20	55.0	7.3	488	480	184
5	15	10	40.6	1.9	384	296	136
6	15	10	46.7	18.8	424	368	184
7	15	10	67.0	12.6	476	384	216
8	15	10	70.4	0	560	376	276
9	14	20	93.0	4.9	576	460	296
10	20	10	49.5	19.0	416	344	216
11	25	10	72.5	27.9	460	380	346
12	25	10	67.8	20.4	508	372	380
13	25	10	105.5	29.0	564	424	470

From Table I it will be noted that for pieces of the same length the volume of the stem varies at different levels because the stem is narrower at the base. It might be argued that the reduction in volume of the stem was responsible for the reduction in rate of regeneration. The volume of the most distal 5-mm. piece is $39.4 \cdot 10^7\ \mu^3$, while the most proximal 5-mm. piece has a volume of only $26.0 \cdot 10^7\ \mu^3$. This is a small variation in volume compared to the change in rate from 71.6 R. U. to 4.2 R. U.

The Effect of Length of Stem on Rate of Regeneration of the Ends

Figure 3 shows the effect of varying the volume of the stem but keeping the level of the cut constant; i.e. using the same region of the stem but with more stem attached to it. This is accomplished by a series of ligatures as in Fig. 1, *F*, *G*, *H*. Inspection of Fig. 3 tells us that the rate of regeneration at a particular level is increased by increasing the volume of the stem but that the increase is small. For example, increasing the volume from 20.8 to 155 units produces a change in rate from 22 to 26 regenerative units for the proximal hydranths. Further increase of volume to 312 units increases the rate to 34 regenerative units. The distal hydranth behaves similarly.

TABLE III

Comparison of relative and absolute rates of regeneration. D^r = relative rate in $\mu^3/\text{hr}\cdot 10^5$; D^a = absolute rate. Same for proximal. Dominance $= \dfrac{P^a - P^r}{P^a} \cdot 100.$

Experiment	No. Stems	Length	D^r	P^r	D^a	P^a	Dominance
		mm.					*per cent*
A	6 whole stems	5	45.5	0	—	36.8	100
B	14 distal halves	7.5	40.5	0	38.6	31.9	100
C	14 proximal halves	7.5	23.4	3.9	24.9	15.1	74
D	10 distal thirds	10	52.7	10.7	57.0	35.9	70
E	10 middle thirds	10	36.1	4.4	26.0	13.0	66
F	10 proximal thirds	10	19.1	0	27.0	8.6	100
G	10 distal halves	13	86.7	41.7	90.5	53.0	22
H	10 proximal halves	13	41.4	17.9	53.6	28.5	38
I	10 whole stems	15	67.0	12.6	63.0	26.8	54
J	10 whole stems	15	46.7	18.8	40.4	20.4	7
K	10 whole stems	20	49.5	19.0	51.2	28.9	34
L	10 whole stems	25	105.5	29.0	108.0	47.4	39
M	10 whole stems	25	72.5	27.9	66.8	34.0	20

Compare these small changes in rate with the increase obtained with change in level (Fig. 2). It is of interest here that the total increase in rate for the distal and proximal hydranth, when the 312 units of stem are used, is the same and amounts to about 12 units. This value may express the amount which the middle of the stem contributes to the ends in regeneration.

The Relative Rates of Regeneration of Pieces of Tubularia

Knowing the differences in inherent rates of regeneration and also the effect of volume on rate, we are in a position to study relative rates and the factors that determine these rates. The rate is termed "relative rate" whenever both ends of a piece of stem of *Tubularia* are allowed to regenerate without a ligature. (See Fig. 1; *A*, *B*, *C*, *D*.) The superscript "*r*" is used. In this case two hydranths are regenerating and are doing so in relation to one another, since they are connected by the stem. Here competition and resultant dominance come in, especially with short stems.

Relative Rates of Whole Stems.—In Table II the relative rates of whole stems of different lengths have been recorded. The stems are prepared as in Fig. 1, *A*. The dominance of the distal regenerating end becomes greater as the stem is cut shorter, so that at 5–10 mm. proximal hydranths often do not appear. As the length of the stem is increased, the rate of regeneration of the proximal hydranth increases. Other factors, such as thickness and volume of stem, play a part.

Relative Rates of Regeneration in Distal and Proximal Halves.—When the stem of *Tubularia* is cut into halves, four hydranths may develop, which we designate as D_1, D_2, P_1 and P_2, as in Fig. 1, *B*. The rates for such stems of various lengths are given in Table III. To show the steepness of the gradient and also the phenomenon of dominance, the relative rates are plotted in Fig. 4. In long stems (26 mm.), there is very little difference in the relative rate of regeneration of the distal hydranth of the proximal half, D_2, and the proximal hydranth of the distal half, P_1. This is to be expected if there is no dominance, since these are adjacent levels of the stem (Fig. 1, *B*). The rapidly developing distal hydranth, D_1, in the distal half does not inhibit the proximal hydranth, P_1, any more than the slowly developing proximal hydranth, P_2, in the proximal half inhibits the distal hydranth, D_2. There is thus no apparent dominance exerted in the ordinary sense, i.e. as an inhibition exercised by a more rapidly regenerating region.

However, in shorter stems dominance appears, and the relative rates of regeneration of the distal hydranth of the proximal half, D_2, and the proximal hydranth of the distal half, P_1, are then quite different. In Fig. 4 the rate of $D_2 = 23.4$ R. U., $P_1 = 0$. In the distal half the distal hydranth D_1 is completely dominant over the proximal P_1. In the proximal half of the stem the distal hydranth, D_2, is only partially dominant over the proximal hydranth, P_2. The rate of $P_2 = 4.0$ R. U.

Finally, in Fig. 4, we have plotted the rates of regeneration of the six possible hydranths, D_1, D_2, D_3 and P_1, P_2, P_3 of Fig. 1, *C* for 10-mm. lengths of stems originally 30 mm. long. The graded differences in rate of regeneration in different regions of the stem are shown by the rates of the distal hydranths and also by the proximal hydranths in Table III, Experiments *D*, *E*, *F*. It will be noted, however, that at any level at which stem is cut the distal hydranth of that level regenerates much more rapidly than a proximal hydranth at the same level. This means that the distal regenerating hydranths in each third of the stem are inhibiting the proximals in that same third. The situation can be summarized by saying that $D_1^r > D_2^r > D_3^r$ and $P_1^r > P_2^r > P_3^r$, but that $D_2^r > P_1^r$ and $D_3^r > P_2^r$, showing that both D_1 and D_2 are dominant. P_3^r is 0, showing the dominance of D_3.

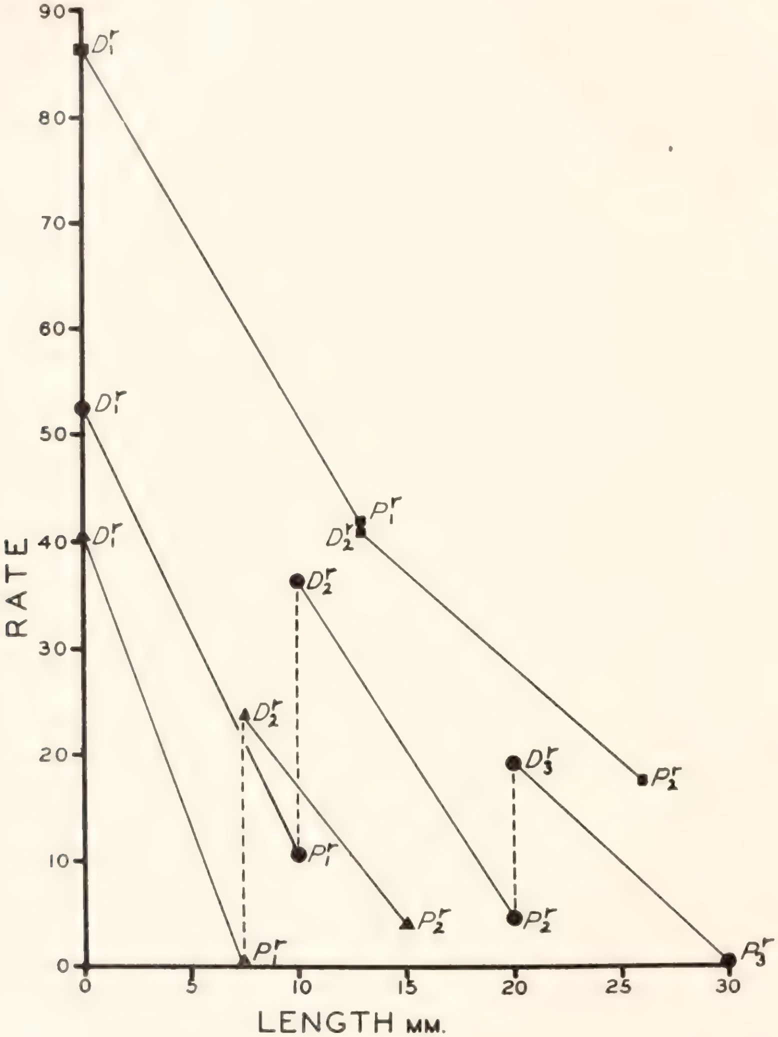


FIG. 4. Relative rates of regeneration of cut stems. Squares = stems 26 mm. long cut into halves; triangles = stems 15 mm. long cut into halves; circles = stems 30 mm. long cut into thirds. D_1^r, D_2^r, P_1^r, etc. = relative rates of respective hydranths. Rate = μ^3/hrs·10^6. The dotted line serves to connect adjacent regions of a cut stem.

The plot of rates in three sets of stems in Fig. 4 shows quite clearly that in each case the gradient is steeper in the upper (distal) levels of the stem. The slopes of the gradient in the upper levels of the three sets of stems are approximately parallel, which seems to indicate that the drop in rate per unit length of stem is about the same in different stems. It should be pointed out that the gradient here will be steeper than the gradient in inherent rates (Fig. 1), since dominance lowers the rate of proximal hydranths.

The Reciprocal Influence of Two Regenerating Regions

In stems of *Tubularia* from 10 to 30 mm. in length, a hydranth usually regenerates at both ends, and it is the purpose of the following experiments to show how the rate of regeneration changes when either the distal or proximal hydranth is prevented from regenerating by means of a ligature allowing the hydranth at the opposite end to regenerate free from dominance. These rates have been termed absolute rates, D^a, to distinguish them from relative rates, D^r. When only one end of a stem is allowed to regenerate, the rate is termed "absolute rate" in contrast to relative rate. This absolute rate of one end can be determined by simply tying off the opposite end of a stem, which prevents regeneration at this end. This rate tends to be the maximal rate of regeneration of a given region since the region has the entire length of the stem affecting its rate and is also independent of a second regenerating region (Fig. 1, *H*, D^{22}). It will also be seen that the absolute rate is simply the inherent rate plus the increase due to the addition of the stem.

The Influence of the Distal Regenerating Region on the Proximal End.—In general (Table III), the shorter the stem the greater the inhibiting effect of the distal regenerating region, and the following cases are arranged according to the length of the stem. In each experiment three lots of stems were used. The first lot regenerated without a ligature, giving relative rates D^r and P^r. The second lot regenerated with the distal end tied off, giving the absolute rate of the proximal end P^a, while in the third group the proximal end was ligatured, giving the absolute rate of the distal end, D^a.

Without exception (Table III), the absolute rate of any proximal hydranth is higher than its relative rate. Indeed, in some cases the relative rate may be 0, while the absolute rate is fairly high, 36.8 units (Experiment *A*). This method of treating the proximal end furnishes a measure of dominance, for if we know the absolute rate and the relative rate we can calculate the percentage reduction due to the distal end, i.e. the inhibition exercised by a dominant region. In

Table III, last column, this percentage has been calculated, and dominance varies from 100 per cent in short pieces to 7 per cent in longer pieces.

The Influence of the Proximal Regenerating Region on the Distal End.—In contrast to the proximal hydranth, the distal hydranth is affected little if at all by the ligature at the proximal end. This is true in the upper levels of the stem. Thus, in Table III, Experiment *B*, the relative rate of the distal hydranth, $D_1^r = 40.5$ R. U., while the absolute rate of the same hydranth, $D_1^a = 38.6$ R. U. and also, Experiment *L*, $D_1^r = 105.5$, $D_1^a = 108.0$. However, in lower levels of the stem, the absolute rate of the distal hydranth is usually greater than the relative rate. Experiment *F*, the relative rate of the distal hydranth of the proximal third of the stem $D_3^r = 19.1$, the absolute rate of the same hydranth, $D_3^a = 27.0$; Experiment *H*, $D_2^r = 41.4$, $D_2^a = 53.6$. This is extremely interesting, because it shows that the proximal hydranth does have an inhibiting influence over the distal hydranth in lower levels of the stem. In proximal regions of the stem then there is a reciprocal influence of the two regenerating ends on each other, each tending to inhibit the other.

TABLE IV

The relation of the inherent rates of regeneration to the relative rates in pieces of *Tubularia* of varying length. Rate $= \frac{\mu^3}{t} \cdot 10^5$. D^i = inherent rate of distal hydranth; P^i = inherent rate of proximal hydranth; D^r = relative rate of distal hydranth; P^r = relative rate of proximal hydranth.

Length	Ligature D^i	Ligature P^i	No Ligature D^r	No Ligature P^r	Ratios		Remarks
					D^i/P^i	D^r/P^r	
mm.							
25	54.6	22.0	72.5	27.9	2.46	2.60	Chiefly partition, no dominance.
20	38.2	13.9	49.0	19.0	2.74	2.60	Chiefly partition, no dominance.
14	81.5	22.6	93.0	4.9	3.61	19.0	Dominance incomplete.
5*	71.6	47.0	78.0	0	1.53	∞	Dominance complete.

* t_1 used in place of t_2.

The Competition between Two Regenerating Regions Having Different Rates

In the second section of this paper it has been shown that adding parts of the stem to either the proximal or distal end increases the rate of regeneration. Therefore the stem as a whole contributes materials to the regenerating ends, and the way in which this material is partitioned can be studied in stems of different lengths which provide different amounts of the material. The following experiments were designed to determine how the distal and proximal hydranths would divide or partition the effect of adding a definite volume of the stem to the system. For this purpose the inherent rates of regeneration of the proximal and distal hydranth were found by tying 3-mm. ligatures at the ends of the stems as in Fig. 1, *F* and *H*, $D_1{}^i$ and $P_1{}^i$. In the same stems the opposite ends $D_1{}^{22}$ and $P_1{}^{22}$ give the rates of regeneration with 19 mm. of stem added to each end. Finally, as in Fig. 1, *A*, the two ends were allowed to regenerate in competition for the intervening 19 mm. of stem.

It is seen from Table IV that in long stems (20–25 mm. lengths) the addition of the middle of the stem to the distal and proximal ends increases the rate of regeneration of these ends in direct proportion to their inherent rates. That is, the relative rates are directly proportional to the inherent rates $D^r/P^r = D^i/P^i$. Therefore there is simply a partition of materials without any dominance. This result would be expected if no factor other than available foodstuffs controlled the rates of regeneration.

TABLE V

Increase in rate of regeneration of distal and proximal ends when the same volume of stem is added to each.

Total Length	Stem 3 mm. Inherent Rate		Volume Added $\mu^3 . 10^7$	Stem 22 mm. Absolute Rate	Increase in Rate
mm.					
25	Distal	54.6	280	66.8	12.2
	Proximal	22.0	266	34.0	12.0
	Stem 3 mm. Inherent Rate			Stem 17 mm. Absolute Rate	
20	Distal	38.2	163	51.2	13.0
	Proximal	13.9	176	28.9	15.0

However, in short stems (14 and 5 mm. lengths) there is no longer a partitioning of substances in proportion to the inherent rates of the distal and proximal hydranths, but rather a dominant effect of the distal end, so that it takes more than its share. $D^r/P^r > D^i/P^i$. As a matter of fact, in all non-ligatured 5-mm. pieces examined in this experiment and many others, no regeneration occurred at the proximal end at all, in spite of the fact that the proximal end had an inherent rate almost as great as the distal $D^i/P^i = 1.53$. This situation represents complete dominance in which all of the material goes to the distal hydranth.

A Comparison of the Effect of the Distal Half and the Proximal Half on the Inherent Rate of Regeneration of the Ends

It has been shown that the middle regions of the stem contribute materials for the regeneration of the two ends. The amount which the stem contributes can be measured by the increase in the rate of regeneration of the hydranths under the assumption that rate is proportional to amount of substances available. Thus, in Table V, the inherent rate of regeneration of the distal end is 54.6 R. U., and when we add 19 mm. of stem, the rate increases to 66.8 R. U. or an increment of 12.2 R. U. Similarly, P^i increases from 22.0 to 34.0 R. U., or an increase of 12.0 R. U. The increase is the same for both proximal and distal ends. (See 20-mm. stems also.) It is clear that the materials of the stem can be used by either the proximal or distal end.

Now if we ligature the stem in the middle, the effect of the materials in distal and proximal halves on the regeneration of the ends may be studied (Table VI). It is found in the two experiments available that the increase in rate is much greater in the distal as compared with the proximal half. This may be taken to mean that there are more materials available in the distal half than the proximal. The curves for the effect of volume on rate (Fig. 3) also indicate that the distal half is more effective in increasing rate than the proximal. As volume is added to the distal end, the rate goes up sharply and then falls off. However, as volume is added to the proximal end, the rate goes up slowly at first and then sharply. The evidence seems rather conclusive that the two regions differ in their effects on rate of regeneration.

TABLE VI

Comparison of the effect of distal and proximal halves of a stem on the rate of regeneration of the ends. D^i is the inherent rate of regeneration of the distal end. Under distal half is the rate of the distal end, with middle ligature. Similarly P^i is the inherent rate of the proximal end. Proximal half = rate of proximal end with middle ligature.

	D^i	Distal Half	Increase	P^i	Proximal Half	Increase
25 mm.	54.6	64.6	10	22	26	4
14 mm.	82.0	98.0	16	22.6	24.2	1.6

Isolation of Distal and Proximal Regions by Means of a Middle Ligature and its Effect on the Relative Rate of Regeneration of the Ends

This method of studying dominance was used in the first experiments, and it is complicated by the fact that not only is isolation produced by the ligature but the volume of the stem is also reduced. The effect on the proximal hydranth is a dual one. Isolation increases the rate of regeneration, while reduction in volume decreases the rate, and the effect is a summation of the positive and negative action. Since, as we have shown, the reduction of the volume of the stem adjacent to the proximal hydranth decreases the rate to a small extent only, the chief effect is to remove the dominant region from the sphere of action, and the net result is an increase in the rate of the proximal hydranth (Table VII).

Length is an important factor, as in 25-mm. stems the change in rate with ligature is slight, while in 15-mm. stems it may be much greater, depending on the condition of the stems. This is in keeping with the fact that little dominance is exerted in long stems.

TABLE VII

Rate of regeneration of distal and proximal hydranths of *Tubularia* with and without a middle ligature. D^r and P^r = relative rate; D^l and P^l = rate under conditions of middle ligature.

Experiment	Length	No.	D^r	D^l	P^r	P^l
	mm.					
1	25	10	67.8	70.5	20.4	25.4
2	25	10	72.5	64.6	27.9	26.0
3	15	10	46.7	43.2	18.8	21.0
4	15	10	63.0	64.0	17.5	23.0
5	15	10	40.6	36.4	1.9	15.0
6	15	10	47.6	38.6	0	15.1
7	14	20	93.0	98.0	4.9	24.2

The Mechanism of the Dominance Exerted by the Distal Regenerating Hydranth over the Proximal End of the Cut Stem

In all previous experiments a ligature was used to block the dominance exerted by the distal regenerating end over the proximal end. With ligature of the stem the proximal end is allowed to regenerate independently of the distal and its rate of regeneration is greatly increased. This ligature, however, not only stops circulation between the distal and proximal end but also severs cellular connections. Thus it is not clear whether the factor responsible for dominance is something present in the circulation or something transmitted or transported through the cells. Therefore it is necessary to determine the effect on the proximal end of stopping circulation from the distal end but leaving cellular connections intact.

Use of a Loose Ligature for Blocking Circulation.—It is not easy to shut off circulation between parts of the stem of *Tubularia* as the cells will rearrange themselves after compression of the stem so that the circulation breaks through once more. However, there are indications which can be seen from Table VIII where the effects of a loose ligature which was tied so as to just stop circulation is compared with a tight ligature cutting through the cœnosarc and breaking all cellular connections. The control with no ligature shows that we are dealing with stems in which the distal end is almost completely dominant over the proximal end: i.e. distal end, 38.7 R. U.; proximal end, 2.9 R. U. A tight ligature completely isolating the two halves sends the rate of regeneration of the proximal end up to 23.0 R. U. or an 8-fold increase. The distal end shows a slight reduction to 34.0 R. U. as it is cut off from the proximal half of the stem. Now with a loose ligature where cellular connections are still intact the proximal end shows an increased rate of 23.0 R. U. over controls in spite of the fact that at the end of the experiment circulation was reëstablished in a few cases through the ligature. This sort of experiment, while it appears conclusive, is not entirely satisfactory as it is difficult to control the tying of a ligature so as to cut off circulation without breaking cellular connection between the two halves.

TABLE VIII

Rate of regeneration of distal and proximal ends of *Tubularia* under conditions of ligature in the middle of the stem. Rate = L/t_2 where L is length of primordium in micra and t_2 is time in hours from cutting of stem to emergence of hydranth.

No. and Length of Stems		No Ligature		Tight Ligature		Loose Ligature	
		D	*P*	*D*	*P*	*D*	*P*
10	15 mm.	38.7	2.9	34.0	23.0	36.4	23.0

Injection of Oil to Block Circulation.—The stem of *Tubularia* is about 0.5 mm. in diameter and it is relatively easy to insert a micropipette for injection. It is necessary merely to crack the rigid perisarc first with two pairs of sharp watchmaker's forceps after which a pipette can be inserted while observing under a binocular microscope. A small drop of paraffin oil (Nujol) is injected and after the pipette is withdrawn the rigid perisarc snaps back into place. The perisarc must not be removed because then regeneration will take place at the exposed surface. Controls for this type of experiment were stems in which the perisarc was ruptured and the pipette inserted without injection. Some of the experiments where oil was injected also served as controls since the drop was sometimes too small to block circulation.

Table IX records results. There are 25 stems in each sample and of the 25 controls only 2 proximal hydranths developed, making the rate 4.65 R. U. and thus showing that the distal end exerted rather complete dominance. When circulation is blocked, however, the 25 injected stems regenerate 12 proximal hydranths, bringing the average rate to 22.8 R. U., or a 5-fold increase in rate. The distal hydranth in the injected group shows a small decrease in rate, as might be expected from previous results on the use of ligatures. A ligature in a short stem increases the rate of the inhibited proximal end but decreases the rate of the dominant distal end by shortening the stem.

Only 12 out of 25 possible hydranths appear at the proximal end of injected stems and it is interesting to examine the 13 stems which did not form a hydranth proximally. Of these 13 negative cases 9 showed that the oil drop had moved from its original position at the middle of the stem into the distal end. In the 12 stems which form a proximal hydranth all but 2 showed the oil in the original position. It is clear that because the size of the oil drop varies the smaller drops do not completely block circulation and as a result they are carried by the circulation to the distal end. When the drop is larger it is held firmly in place by the endodermal lining of the gastrovascular cavity and in these cases proximal hydranths appear associated with a complete block to circulation.

TABLE IX

Injection of an oil drop into the gastrovascular cavity of *Tubularia*. Stems 6 mm. long. Twenty-five stems used in each sample. R = rate of regeneration $= \pi r^2 L/t_2$ where r = radius of stem in micra; L = length of primordium in micra and t_2 = time in hours required for emergence of the newly regenerated hydranth.

	Oil Injected		No Injection	
	Distal	Proximal	Distal	Proximal
L.	1384	528	1536	104
r.	282	264	284	280
t_2.	40.0	50.5	40.9	55.0
R.	86.1	22.8	95.0	4.65

In other experiments where dominance is not so complete there is a quantitative effect on rate of regeneration of the proximal end, with injection of oil into the gastrovascular cavity. It must be remembered that this effect is not so great as would be expected since not all of the oil drops are large enough to block circulation. An example is shown in Table X, where the proximal ends of control stems regenerate at a

TABLE X

The effect of blocking circulation in *Tubularia* with oil drops. Oil injected into distal region. Controls consist in injury similar to that of injection. Twenty-one stems, 10–11 mm. in length used for each sample. $R = \pi r^2 L/t$.

	Oil Injected		Injury Control	
	Distal	Proximal	Distal	Proximal
L	1200	1032	1352	612
r	254	236	252	228
t_1	35.4	47.0	37.3	50.0
R	67.0	38.4	72.0	20.0

rate of 20.0 R. U., but upon injection of oil increase to 38.4 R. U. The rate of the distal hydranth is reduced from 72.0 R. U. in untreated stems to 67.0 in injected stems. This decrease is in part caused by injection of the drop into the distal region of the stem which isolates a small portion. The average length of stem from the oil to the distal end was 2.5 mm. at the termination of the experiment.

TABLE XI

Effect of blocking circulation by means of oil in *Tubularia*. Stems 8–10 mm. long isolated from long thick stems. Twenty-eight stems in each sample. R = rate using t_1; l = length of primordium in micra; r = radius of stem in micra; t_1 = time in hours from cutting to formation of primordium. Rate = $\mu^3/\text{hrs.} \cdot 10^5$.

	Oil		Control	
	Distal	Proximal	Distal	Proximal
L	1388	884	1320	852
r	286	276	286	268
t_1 hours	37.8	46.9	37.3	46.0
R	94.0	45.0	91.0	41.7

Finally, in stems where there is very little dominance there is little effect of injection of oil in the middle of the stem. This result was obtained from some 8–10 mm. pieces cut from long, thick stems. Table XI shows that neither the proximal nor distal hydranths are affected to any extent by the injection of oil. This result is to be expected from the section dealing with the use of a middle ligature to isolate the two ends. In stems where the distal hydranth exerts little dominance it was found that little effect was produced on the rate of regeneration of the proximal hydranth by a ligature.

The experiments on injection of oil into the gastrovascular cavity of *Tubularia* shows that the oil isolates the proximal end of the stem from the distal end, producing about the same effect as a ligature. In the former case the circulation is blocked while in the latter both circulation and cellular transmission are blocked. It becomes important then to see just what the oil drop does in the gastrovascular cavity. It has already been pointed out that when the drop is small it has little effect in blocking dominance. Also, when there is little dominance, little or no effect of injection of oil is found. Therefore it is safe to say that there are no toxic chemical or physical effects of the oil on the cells which come in contact with the oil.

Examination of the region into which the oil is injected shows it to be firmly held in place by the endoderm which it partially displaces. When it is not so held in place it moves during the course of hours to the distal end of the stem. Although the endoderm is displaced and perhaps the cellular connections in this layer are broken, the ectoderm remains intact and the cells can be seen to be continuous over the surface of the oil drop. As we were not satisfied with this observation, the conductivity over the bridge of ectoderm was tested by using an electrical stimulus. In some previous unpublished work on electrical stimulation in *Tubularia* it was found that upon applying a stimulus at the proximal end the tentacles of the distal hydranth would respond. Three stems in which dominance was blocked by injection of oil were treated in this manner and in each case the tentacles of the distal hydranth responded to an electrical stimulus applied at the proximal end. From these observations there can be little doubt that the ectodermal connections over the oil drop are morphologically and physiologically intact and that dominance is not transmitted over this layer.

Discussion

Child (1907) pointed out that in *Tubularia mesembryanthemum* both the length of the cut stem and the level at which the stem was cut were factors determining the time for regeneration and size of the primordium. Driesch (1899) before this had measured the primordium of halves of stems and found that the oral half (distal half) formed longer primordia than the aboral (proximal half). Driesch also showed that the hydranths emerged faster in the oral (distal) half as compared with the aboral (proximal) half. Thus the regional differences in regenerative capacity in the stem of *Tubularia* are by no means new. The new treatment of the facts by combining two variables, size and time, into a rate has not been suggested hitherto. By utilizing both variables it is possible to express the rate of change within the stem at any level and so compare rates under various conditions. It is hoped that the rate as defined in this paper is a measure of the chemical changes involved in the differentiation of the hydranth from the stem after cutting. It is not sufficient to express these changes in terms of time only since two hydranths of different sizes may regenerate in the same time and certainly the larger hydranth must have utilized more material than the smaller. Therefore the rate of chemical change must have been higher in the region which formed the larger hydranth. Similarly two hydranths of the same size may require different times for regeneration.

A second difference between these experiments and those of early investigators is the use of a ligature to isolate regions in order to test their rate of regeneration. Driesch (1899) and Child (1907) cut stems into halves and thirds and allowed both ends to regenerate. The size and time for regeneration of distal (oral) hydranths is modified by the presence of a second region of regeneration. Both investigators showed that the size and time factors varied with the length of stem cut. In my experiments by the use of the ligature the second regenerating region is eliminated and the size kept constant. Thus the "inherent" rate is measured. It is proposed that the "inherent" rate of regeneration be used as a base so that the effect of variables such as length of cut stem and the rate of regeneration of a second hydranth can be studied by means of appropriate ligatures.

Since the stem of *Tubularia* shows a gradient of "inherent" rates there must be graded differences in the concentration of some substance or substances, which account for these different rates. Further, because the rates are higher in the younger (distal) regions of the stem it is reasonable to assume that the substances are of the nature of a synthetic factor which is able to convert available materials into a hydranth.

We will let this inherent synthetic factor be represented by E and assume that the concentration of E is proportional to the inherent rate of regeneration as measured by isolation of parts of the stem. E is then present in highest concentration in the young cells at the distal end and in lowest concentration at the proximal end. Then Fig. 2, giving rates of regeneration, may be taken to indicate the relative concentration of E at various regions of the stem since only internal factors are responsible for these differing rates at various levels of the stem.

But E is not the only factor affecting rate. In Fig. 3 it was shown that increase in length of stem adjacent to the regenerating region will also increase rate of regeneration. It is evident that something from the middle of the stem travels to the ends and causes an increase in rate. Let us call this factor or substance S. S is transported through the gastrovascular cavity in the circulation which is easily observed in *Tubularia*. A cross-section of the stem shows four channels in the endoderm and in the intact stem particles can be seen travelling up one side of the stem and back down the other so that a fairly rapid circulation exists. Timing the flowing particles gave a velocity of 6 mm./minute. This means that in a short stem 6 mm. long a complete circulation of the contents of the coelenteron should take place in 2 minutes. If one end was using up materials in rapid regeneration it is conceivable that the concentration of substance, S, in the circulation would be lowered considerably so that at the opposite end substances might pass into the gastrovascular cavity from the cells and so inhibit regeneration by removal of available materials.

The effect of S on rate of regeneration is difficult to measure but we may take Fig. 3, which shows increase of rate with increased volume of stem as a provisional measure of S. Obviously, however, this does not give us the effect of S in very low concentrations. That S is a very important factor is seen by comparing the inherent rate of the proximal end with the relative rate (Table IV, 14-mm. and 5-mm. stems). In these cases something is actually removed from the proximal region so that although the stem is much larger the rate of regeneration is lower when the distal hydranth is regenerating.

The situation may be summarized as follows. Regeneration is essentially the transformation of stem into hydranth and this requires chemical changes: $S \overset{\overset{E}{\downarrow}}{\rightleftarrows} H$. Let us assume E to be a catalyst in the cell which transforms S into H, H being the substances necessary for hydranth differentiation. The reaction is reversible, as Child (1923) has shown that hydranths may dedifferentiate into stem. I have also observed a hydranth partially differentiate from cœnosarc and then return to cœnosarc. E, as we have pointed out, is present in the cells and is in highest concentration at the distal end. S is in the cells and also the gastrovascular cavity and is present in greatest amount in the longest stems.

In long stems, where S is high (Table IV, 25-mm. and 20-mm. stems) it appears that S is partitioned to the distal and proximal hydranths according to their inherent rates of regeneration, as represented by E. One way of expressing it is that, with increase in S while Ep (concentration of E at the proximal end) and Ed (concentration of E at the distal end) remain constant, H_p (concentration of H at proximal end) and H_d (concentration of H at distal end) increase proportionally. This result is the expected one.

The difficulty comes when we consider short stems, where E_p (concentration of E at the proximal end as measured by the proximal inherent rate) is close to E_d (concentration of E at the distal end as measured by the distal inherent rate) yet apparently S is used by the distal hydranth and not by the proximal hydranth. All that can be assumed is that the factor E_d converts S into H as fast as it appears in the cœlenteron, so that the concentration of S is always below the minimal value necessary for regeneration at the opposite end. Since $E_d > E_p$ it can synthesize H at a lower concentration of S. By writing the reaction as a reversible one, we may even suggest that $H \rightarrow S$ at the proximal end in the case of short stems. This whole thought depends on the assumption that the difference between E_d and E_p in short stems, although small, is great enough to lower the concentration of S below a minimal value, S_m for the proximal hydranth. As S increases in amount (in longer stems) the concentration in the cœlenteron rises above the minimal value S_m for the proximal hydranth and regeneration starts at the proximal end though at a low rate at first. S thus becomes a limiting factor for regeneration in low concentrations.

It will be seen that the formal explanation given above is readily applied to other results, such as those from middle ligatures and the results showing the difference between relative and absolute rates of regeneration.

From the comparison of the absolute rate of the distal hydranth D^a and the relative rate of the same hydranths D^r in Table III, it is apparent that there is a maximal rate of regeneration for the distal hydranth, or a maximal concentration of S above which synthesis of H is not increased perceptibly. This is not unlikely.

The above discussion throws the entire effect of dominance on the transport of available substances (S) for regeneration. In *Tubularia* the transport system is extremely simple, and it seems an ideal system for experimentation. If in short stems the circulation of S is blocked (as by an oil drop) both the distal and proximal ends should regenerate independently of each other and no dominance will be exerted by the distal end. The experiments in this paper indicate that such is the case and thus show that S is a factor which circulates in the fluid of the gastrovascular cavity.

Summary

1. The rate of regeneration of *Tubularia* has been measured by the formula $R = \pi r^2 L/t$ where r = radius of cross-section, L = length of regenerate, t = time in hours for formation of primordium or the time in hours for emergence of the fully formed hydranth.

2. The rate of regeneration of isolated parts of the stem decreases

rapidly from the distal to more proximal regions. An increase in the length of stem adjacent to a regenerating end increases the rate of its regeneration.

3. When two regenerating regions are competing they partition the effect of adding the middle region of the stem if the stem is long. If the stem is short the distal end becomes dominant and inhibits the proximal end.

4. A method has been devised for measuring dominance by expressing it as the percentage reduction in rate of regeneration due to the presence of the distal hydranth.

5. The circulation within the stem of *Tubularia* has been blocked by means of injection of a drop of oil with the result that the dominance (inhibition) exercised by the distal regenerating end over the proximal end is blocked.

6. A generalized mechanism based on a synthetic factor E in the cells and a circulatory factor S is suggested as a formal explanation of the phenomenon of dominance.

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CYTOLOGICAL INVESTIGATIONS OF COLPODA CUCULLUS

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Inasmuch as the various species of *Colpoda* have interested many previous investigators, especially with regard to factors of encystment and excystment, it was thought worth while to make a careful study of the best known species, *Colpoda cucullus* Muller. So many interesting and hitherto unreported phenomena have been observed that it was thought advisable to present only the cytological results in this first report. Subsequent reports will deal with the many and interesting observations on other phases of the problem which are at hand and these will be supplemented by further and more complete data.

The life histories of various species of *Colpoda* have been investigated from time to time starting with the work of Stein in 1854. He reported the encystment of *Colpoda cucullus*, the subsequent division into two, four, eight and even sixteen smaller individuals and their ultimate escape from the ruptured cyst. Rhumbler (1888) made an extended study of the process of encystment and division in *Colpoda cucullus* and *C. steini* and distinguished between the division cysts, "Theilungscyste," and those cysts within which division does not occur, the permanent cysts, "Dauercyste." He describes in some detail the appearance and activity of freshly encysted and dividing forms, noting that the cilia are retained throughout the division process but are lost during their stay in the permanent cysts. He observed quite accurately the gradual loss of the food inclusions, during permanent cyst formation, although his interpretation of the method of this loss is open to serious question.

Wenyon (1926) has given a rather diagrammatic account of the division of *Colpoda steini*, agreeing with the accounts of Stein and Rhumbler but adding some details of the nuclei. Wenyon's observations were made on fixed and stained material while those of Stein and Rhumbler were mostly obtained from the living material.

Very recently Penn (1937) has reported the occurrence of binary and quadruple division without encystment in a strain of *Colpoda cucullus*. Encystment before division occurred in his race only when the cultures were old or when the cultures became crowded. He was able to induce the formation of cyst walls by placing "healthy individuals" in infusions containing gelatinous masses of bacteria. In this report are given descriptions of the nuclear phenomena which take place during the divisional process, but details are lacking.

There has been no cytological report of the nuclei of the "permanent" or resistant cysts published to date, as far as we are aware, and the reports of the nuclei during division have all been fragmentary. It is not difficult to see why resistant cysts have eluded observation in the past when one considers the fact that most nuclear investigation has been done after the employment of the various hæmatoxylin stains. As pointed out by Goodey (1913) the cyst walls ("ectocyst and endocyst") stain intensely with both Heidenhain's and Delafield's hæmatoxylins. We have found it impossible to study the contents of the resistant cysts after treatment with these standard stains and no doubt previous workers have experienced like difficulty. It is with the aid of the nucleal reaction developed by Feulgen and Rossenbeck (1924) that the details of the nuclear complex of resistant cysts have been rendered observable.

Hæmatoxylin stains do not react on the division cyst wall as they do on the resistant cyst walls, mainly because of the comparative delicacy of the former. It is possible to obtain a rough idea of what happens to the nuclei during division by employing these stains. But because of the densely packed food inclusions in the cytoplasm during this period fine details are obscured. After the Feulgen reaction, however, the history of the nuclear components may be readily followed. It has been found that this history is a rather surprising one and one that may shed considerable light on the rôle of the macronuclear chromatin in ciliate metabolism. Therefore, because we have observed with considerable exactitude the nuclear phenomena both during division and during the stay within the resistant cyst, and because we feel that these observations will contribute to our understanding of related phenomena among ciliates in general, we offer the following cytological report as the first one of a comprehensive nature on this common organism, *Colpoda cucullus*.

MATERIAL AND METHODS

Colpoda cucullus is a very common form and may be collected in a great variety of places. Our original material was obtained from dry hay taken from the banks of a brackish stream near Stuart, Florida. The hay was placed in spring water from which enormous numbers of the ciliates were later collected and transferred to small dishes. From these dishes a number of motile specimens were selected with a micropipette and placed in individual isolation culture dishes in a drop of twenty-four-hour-old diluted hay tea. After a number of encystments and segregations had taken place one motile ciliate was selected and all of the others discarded. All glassware was then scrupulously cleaned and boiled for a long period of time to remove any danger of contamination. This obvious precaution was to insure the presence of only one species with which to work. All of our material, therefore, has descended from a single organism.

The standard culture medium used in this work, while not the only one successfully used, has given consistent results and the isolation lines grown in it have exhibited a surprising vitality. It consists of nothing more than ordinary hay tea, used after twenty-four hours at room temperatures. Into this medium single motile ciliates were placed and invariably at the end of twenty-four hours three to four divisions had occurred. All that was necessary to obtain resistant cysts was to allow multiplication to proceed for forty-eight hours or longer without adding fresh medium. Under those conditions the division rate decreased until finally all of the ciliates secreted the heavy wall characteristic of the resistance phase, accompanied by the other phenomena described below. Because of the high fission rate, the hardiness of the species and the predictable response to certain environmental conditions there was always an abundance of material in every phase of the life history under investigation.

For the preparation of permanent slides for cytological study we employed special "recovery dishes"[1] for the collection of material designed by one of us (C. L. C.). Small circular flat-bottomed Pyrex dishes with straight sides, measuring 23 mm. × 12 mm. (inside measurements), were prepared. These dishes will accommodate ⅞ inch circular cover glasses, allowing them to rest on the bottom with very little free space about the edges. Into one of the dishes was placed a cover glass, in some cases thinly coated with fresh egg albumin. The dish was then filled with culture medium and inoculated with a single motile *Colpoda*. By frequent microscopic examinations the stages desired could be accurately noted, the cover glass taken out and placed in the fixing fluid. In this way we were able to recover hundreds of the different stages on each cover glass. Another advantage this method has is that the organisms do not tend to pile up but each adheres to the glass more or less separately. There is a minimum of debris which makes for clarity of the final preparations. The success of these "recovery" dishes caused us to give up entirely our earlier methods of concentrating by centrifuging and of selecting individuals under the dissecting microscope.

[1] These dishes, known as the Claff Recovery Dishes, are being put on the market by Clay-Adams Company, Inc., New York City.

As mentioned in the beginning, our clear preparations resulted from the use of the Feulgen nucleal reaction. We made many preparations with the various hæmatoxylins and carmines, and while these preparations were entirely satisfactory for the motile forms and proved very useful, they were mediocre for divisional phases and entirely useless for resistant cysts. We found that certain modifications of the standard Feulgen procedure were advantageous. After much experimentation it was found that the following times gave excellent results and we highly recommend them for future work on *Colpoda cucullus:* acid hydrolysis—15 minutes at 60° C.; fuchsin sulphurous solution—4 to 5 hours; sodium bisulphite-hydrochloric acid wash—15 minutes (at least three changes). It was found that the increased time of washing had a decided tendency to clear the cytoplasm of any trace of free fuchsin and rendered the preparations beautifully clear.

Many types of fixing fluids were used but it was found that wherever cyst walls were present the more penetrating varieties gave, as would be expected, the best results. Therefore most of our material was fixed in Schaudinn's with 5 per cent acetic, corrosive sublimate in 95 per cent alcohol with 5 per cent acetic acid added, or the Gilson-Carnoy mixture. The latter fixing fluid gave the best results on the resistant cysts.

Our Feulgen prepared material was very often counterstained with either fast green in 95 per cent alcohol, methylene blue in 70 per cent alcohol, or the acid component of the Borrel mixture (Calkins, 1930). The last-named stain was modified as to balance from the original, containing ⅓ indigo carmine to ⅔ picric acid. Counter stains reacted well in the cytoplasm of trophic and divisional forms, penetrating the thin cyst membrane of the latter with ease. The resistant cyst membranes appear to be entirely impermeable to fast green, methylene blue and the indigo carmine component of the Borrel mixture. The picric acid of the Borrel stain penetrates very readily, however, staining the cytoplasm an intense yellow. After this counterstain striking preparations are obtained if the material has been taken from a culture in which trophic forms, divisional cysts and newly formed resistant cysts are present. The cytoplasm of the trophic forms and the divisional cysts is a brilliant green while the cytoplasm of the resistant cysts is yellow.

It should be mentioned that numerous preparations employing the silver nitrate method of Klein were made on the trophic forms as an aid to our positive determination of the species at the beginning of the investigation. The extreme variability of the size and form of these ciliates is so great that specific identification becomes difficult without careful study of the ciliary pattern. The question of variation of the ciliary pattern we hope to present in another report.

It has been found possible to check very accurately the times when the various nuclear changes occur during each phase by starting with resistant cysts, inducing them to excyst and fixing material at frequent, timed intervals. In carrying out these timed observations a large number of "recovery dishes" were prepared at one time and their contained organisms fixed in order, first noting the condition of each in the living state. Due to this procedure we have a complete series of slides demonstrating the nuclear activity from excystment, through several reproductive divisions and through a second resistant encystment. As a result of these observations we are certain that the sequence of events to be described is the normal one and occurs in a regular fashion in this ciliate. All the cytological details observed from the timed material have been repeatedly checked from mass cultures.

THE LIFE CYCLE—GENERAL

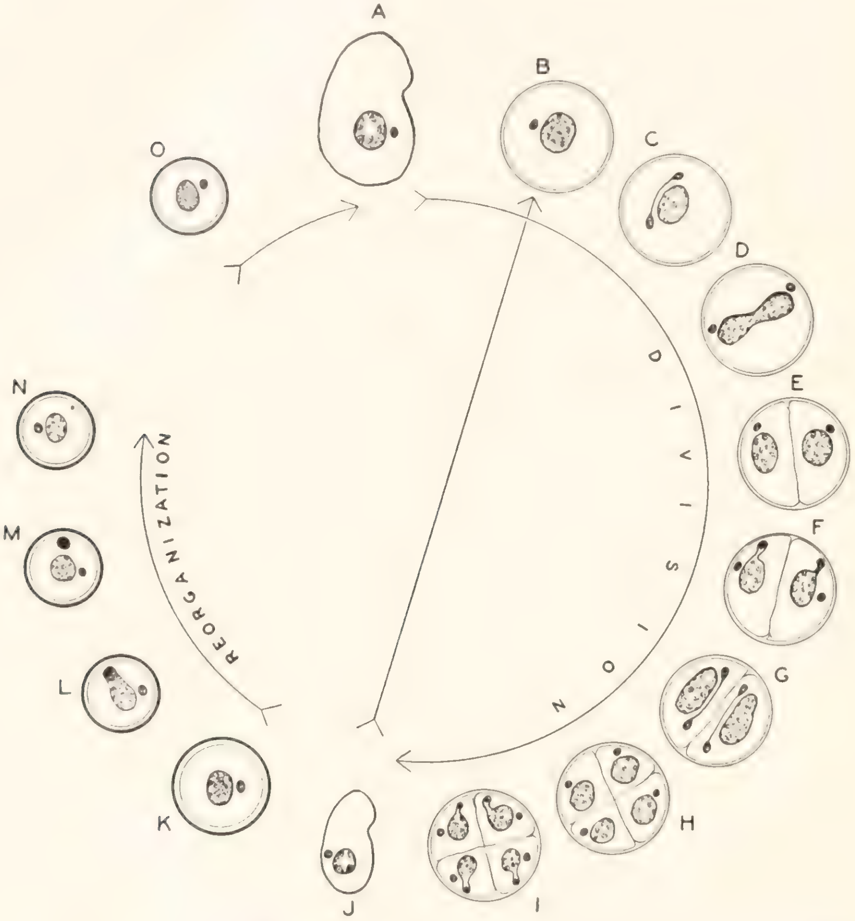
Conjugation has never been observed by us in our strain of *Colpoda cucullus* and as far as we have been able to determine it has never been reported in the literature. Enriques (1908) mentions conjugation as having been noted by him in *Colpoda steini* but not in *C. cucullus*. Therefore we will use the term "life cycle" to denote the sequence of events taking place from one resistant cyst stage back to another.

Colpoda cucullus appears to possess the ability to accommodate itself to a wide variety of environmental conditions. The great factor in this extreme accommodation seems to be its ability to secrete, in a minimum of time, protective or semiprotective cyst membranes. Under normal conditions all reorganizational phases accompanying reproduction and resistant encystment are carried out when the organism is enclosed in some type of membrane or membranes. Feeding, only occurring in the trophic stage, is accomplished in a very efficient manner, for within a few minutes after resistant excystment the cytoplasm will be found to be literally packed with spherical food vacuoles.

When placed in fresh culture medium the resistant cyst undergoes certain changes very rapidly. These changes lead to a rupturing of the heavy outer cyst wall (ectocyst of Goodey, 1913) and finally to liberating the swimming ciliate from the thin inner wall (endocyst of Goodey). One of us (C.L.C.) has been investigating the mechanism of excystment under normal and experimental conditions and will report these findings at an early date.

The newly excysted ciliate is devoid of food vacuoles but very rapidly engulfs great quantities of bacteria and bacterial debris. This food is invariably formed into spherical compact masses and distributed at random throughout the cytoplasm. One average sized ciliate (70 μ) may have as many as two hundred such food vacuoles (Plate I, Figs. 1 and 2). Growth ensues, the ultimate size appearing to be dependent on the excellence of the cultural conditions but independent of the ability to reproduce. Within a few hours after excystment the trophic form begins to round up, without, however, losing the motility of the cilia. The cytostome becomes indistinct and a thin membrane is secreted outside the still moving cilia. This is the "Theilungscyste" of Rhumbler (1888) and undoubtedly corresponds to the "pseudocyst" of *Tillina canalifera* recently described by Turner (1937). In the vast majority of cases quadruple division occurs within this cyst resulting in the liberation of four small daughter ciliates. The cilia may be continually observed throughout the process. Occasionally binary division occurs resulting in the liberation of but two daughter ciliates in which case they are proportionately larger in size. We have never observed divisions resulting in more than four daughters although Stein (1854) has reported as many as sixteen daughter ciliates escaping from a division cyst. The report of Penn (1937), in which he maintains the normal condition in *Colpoda cucullus* to be quadruple division without encystment, was not confirmed in our study. Binary and quadruple division without encystments were encountered only occasionally among the great number of encysted forms irrespective of the bacterial condition of the medium. It is possible that lack of encystment is a peculiarity of the strain studied by Penn.

If no additions are made to the culture medium for a period of forty-eight hours the rate of division cyst formation decreases and stops and the ciliates become very small and less active. They round up very quickly and secrete a heavy, wrinkled cyst wall about themselves. This results in the resistant cyst which is able to withstand desiccation. The formation of resistant cysts may be induced by simply drawing off a part of the medium and replacing it with old medium from a culture in which resistant cyst formation has taken place. In the literature are numerous accounts of the necessity for drying before resistant cyst formation occurs (Barker and Taylor, 1931; Bodine, 1923; etc.). We have found that resistant cysts form readily even in an abundance of fluid, and may be kept for long periods without recourse to evaporation. As pointed out by Penn (1937), if conditions inducing resistant cyst formation occur rapidly enough (evaporation, according to Penn), some of the division cysts become resistant cysts by the simple expedient of forming a heavy wall about the outside of the division cyst wall. Thus resistant cysts may contain one, two or occasionally four cells, all motor organelles dedifferentiating until nothing can be seen but the nuclear apparatus and the granular cytoplasm.



TEXT FIG. 1. Diagrammatic representation of the "life cycle" of *Colpoda cucullus* illustrating the sequence of events during reproduction and resistant cyst formation. *A–J*, normal reproductive activity repeated (*J* to *B*) under favorable cultural conditions. *K–O*, resistant cyst with space between *N* and *O* representing the lapse of an indefinite amount of time while the arrow from *K* to *N* represents a short space of time during which macronuclear reorganization and chromatin elimination takes place. Arrow from *O* to *A* represents the return of favorable conditions for excystment.

A young resistant cyst possesses many food inclusions but these are rapidly absorbed, leaving characteristic refringent inclusions in their stead. Within a few hours even the refringent bodies have disappeared and the cytoplasm becomes compact and evenly granular, containing the nuclear apparatus. The compactness of the cytoplasm is brought about by the actual shrinkage in the size of the organism even after the cyst wall has been laid down. Resistant cysts are always very much smaller than all but the smallest of the trophic forms or division cysts.

The above account agrees essentially with the previously published descriptions of the "cycle" of *Colpoda cucullus* and will serve as a basis for the details of the nuclear activity described below.

Text figure 1 illustrates the normal course of events during the "life cycle" of *Colpoda cucullus* which, with the accompanying legend, will serve to clarify the above description.

The Nuclei of Colpoda Cucullus

Enriques (1908) gives as a specific characteristic of *Colpoda cucullus* the possession of a macronucleus with a lobed karyosome "cariosoma lobato." He was able to observe this after staining with carmine. From his figures it may be assumed that, except for the karyosome, the macronucleus is devoid of stainable material (chromatin). Wenyon (1926) figures the macronucleus of *Colpoda cucullus* with irregular karyosome-like bodies toward the center. He does not state what stain was employed but it would be safe to assume the use of a hæmatoxylin. That both of these observations were due to the type of stain used is demonstrated by a comparative study of organisms stained in borax carmine, Heidenhain's hæmatoxylin and after the Feulgen reaction. In the first two cases the stainable material seems to be concentrated toward the center of the macronucleus surrounded by a faintly stained, irregular periphery. But after the Feulgen reaction the picture is reversed. The chromatin is seen as irregular plaques surrounding and extending into the colorless nucleoplasm (Plate I, Fig. 1). After any of the counter stains used with the Feulgen reaction the nucleoplasm is sharply contrasted to the chromatin and is seen to have the form described as the karyosome by previous workers. Penn (1937) illustrates this general condition in his figures from Feulgen prepared material. We wish to emphasize the chromatin configuration of the macronucleus of the trophic form, therefore, as being in the form of irregular plaques around the periphery and surrounding the non-chromatin nucleoplasm of the center, because of the prevalent use of this character in classification (see the description of Kahl, 1931).

A single micronucleus is always present in the trophic stage. It lies near the macronucleus but may be on any side of it. The chromatin is quite compact and in fixed preparations is usually seen to have shrunken away from the nuclear membrane. The micronucleus is quite commonly elongated, flattened on one surface, and slightly pointed at the ends. We have never found more than the single micronucleus in the trophic forms. Penn (1937) mentions the occasional occurrence of two or four micronuclei in his strain. We believe that it is possible that he interpreted the balls of extrusion chromatin from the macronucleus as micronuclei inasmuch as he completely overlooked these interesting bodies (see our Plate I, Fig. 23).

In our material the micronucleus behaves in an orthodox fashion during the divisional activities. After the division cyst membrane is laid down it enlarges and assumes a spherical shape. The chromatin becomes slightly less compact and is seen to be finely granular (Plate I, Fig. 3). It then becomes distinctly striated and the whole nucleus elongates and enlarges. The striations within the chromatin become more marked and finally irregular threads may be observed all oriented with the long axis of the nucleus (Plate I, Fig. 4). Contraction of the chromatin threads results in the formation of the metaphase plate (Plate I, Figs. 5, 6, 9). There appear to be a great many chromosomes and we were never able to make even an approximate count, as a glance at the figures will reveal. The anaphase is formed by a separation of the chromosomes of the metaphase plate into two groups; the nature of this separation has not been determined. The two daughter chromosome groups move to opposite ends of the fully formed spindle leaving between them definite clear fibers which remain into the elongated telophase (Plate I, Figs. 7 and 8). The chromatin becomes compact again in the daughter telophase groups and these move farther apart (Plate I, Figs. 10 and 19), retaining for some time the connecting strand formed from the nuclear membrane. Finally this strand breaks and the two micronuclei round up and take their positions at opposite ends of the now elongated macronucleus (Plate I, Figs. 11 and 12). This sequence is repeated prior to every cell division without any appreciable variation.

It is within the chromatin of the macronucleus that events occur that offer an interesting problem in ciliate cytology. Before the division cyst membrane is laid down the chromatin loses its plaque-like configuration and becomes flocculent, being roughly dispersed throughout the nucleoplasm. It is still slightly granular but the granules are exceedingly minute (Plate I, Fig. 2). By the time the division cyst membrane is formed the chromatin of the macronucleus has begun to take on a definite configuration (Plate I, Fig. 3), that of granular aggregates suspended in the clear nucleoplasm (Plate I, Figs. 9, 10 and 11). This configuration is retained until the daughter organisms are ready to emerge from the cyst, in most cases through the two divisions. The chromatin aggregates stain uniformly and rather intensely but because of their scattered condition the macronucleus as a whole appears as a loosely knit body, lying among the numerous food inclusions. By the time the micronucleus has reached its telophase stage the macronucleus has begun to elongate. This elongation marks the future division plane of the cell, being always at right angles to it. Further elongation stretches the macronuclear membrane until a typical constriction appears separating the chromatin into two daughter halves (Plate I, Fig. 12). The two daughter halves of the macronucleus quickly separate and round up and the fission plane forms, dividing the cell into two equal daughter cells, each containing a single micronucleus and macronucleus and numerous food inclusions (Plate I, Fig. 13).

Shortly after cell division there becomes differentiated simultaneously within each daughter macronucleus an irregular, granular mass of chromatin. This mass stains much more intensely than the general macronuclear aggregates and is first seen lying close to the nuclear membrane. It is rapidly separated from the chromatin aggregates and is pushed out from the surface of the macronucleus being surrounded by a pocket formed from the macronuclear membrane. This activity is nearly or exactly synchronous in each daughter macronucleus (Plate I, Fig. 14). This deeply staining chromatin mass is the "extrusion chromatin" or "residual chromatin" and is ultimately broken away from the macronucleus and cast into the cytoplasm (Plate I, Figs. 15 and 17). Within the cytoplasm it becomes compacted into an intensely staining ball which rapidly diminishes in size until it disappears from view. It is usual that complete absorption of the extrusion chromatin is accomplished before the start of the second fission.

The second division is initiated, as in the first, by the activity of the micronucleus. All stages appear to be the same as in the preceding division (Plate I, Figs. 18 and 19) with the result that four daughter ciliates are formed within the original division cyst membrane. Occasionally the cyst wall becomes soft and irregular and liberates the two daughters before the second division. Figure 17 represents a case of binary fission just before the liberation of the daughter cells. The extrusion chromatin has not been absorbed yet, and may not be until after the daughters become free-swimming organisms.

Following the second cell division each macronucleus again undergoes a reorganizational process whereby more extrusion chromatin is formed and cast out into the cytoplasm (Plate I, Figs. 20 and 21).

After this process has taken place the four daughter ciliates become more active, the cyst wall becomes softer and more irregular and finally ruptures. In the majority of cases enough time elapses during the freeing process for the extrusion chromatin to become absorbed but sometimes the cyst membrane ruptures early and the young daughter organisms each carry with them the remains of the residual ball (Plate I, Fig. 23). This residual ball might easily be mistaken for a supernumerary micronucleus, as we feel sure has been the case in the report of Penn (1937) on the occurrence of more than one micronucleus.

When, as occasionally happens, as mentioned above, binary or quadruple division takes place without the formation of a definite cyst membrane there occurs exactly the same nuclear activity as found in the normal division cyst. Chromatin extrusion follows each division in as regular and predictable a fashion as that just described. Figure 22 represents the end result of a quadruple division without cyst formation. These daughter ciliates are completely reorganized and all trace of the residual chromatin has disappeared. We have been able to find all stages representing the above process of chromatin elimination in these divisions but because of the duplication of the conditions found during the normal process and because division without cyst formation was the exception in our material we felt that illustrations would be redundant.

The above process of the alternation of a free-swimming, feeding organism with reproduction within the division cyst is repeated every eight hours, on the average, either in isolation or mass cultures so long as fresh medium is provided. Whether or not there will be found a waning vitality over extended periods of time we cannot tell at present. Experiments testing this point are being carried out and will be reported at a later date.

When conditions within the culture change due to the accumulation of waste products resistant cysts are formed. This process is accomplished in a very short period of time and involves the laying down of a thick, relatively impermeable wall, the absorption of the food bodies and the concentration of the cytoplasm. No activity on the part of the micronucleus is observed but the macronucleus proceeds to the most profound reorganization as yet reported for any holotrichous ciliate.

Resistant cyst formation in a trophic organism proceeds with a diminution in size and a rounding up of the ciliate. As the heavy cyst wall is secreted the cilia disappear and the cyst becomes firmly attached to the substrate. The nuclear apparatus is much the same in appearance as in the trophic stage except for the fact that both nuclei become smaller and slightly more dense (Plate II, Fig. 24). The food inclusions are rapidly absorbed and the cytoplasm then contains numerous minute refringent bodies (Plate II, Figs. 25 and 26). After the disappearance of the food balls but before the disappearance of the refringent bodies the macronucleus begins to elongate and to differentiate into two distinct regions. One end becomes more intensely staining than the other as if the chromatin was becoming com-

EXPLANATION OF PLATES

All figures are of *Colpoda cucullus*, and with the exception of Figs. 4–8 the magnification is × 1,000. Figs. 4–8 represent a magnification of × 2,000. All figures are from preparations treated with the Feulgen reagent. Fixatives used were Schaudinn's fluid with 5 per cent acetic acid for the preparations illustrated on Plate I and Gilson-Carnoy fluid for the illustrations on Plate II. The cilia, which are present in all stages represented on Plate I, have been omitted from the illustrations. Asterisks (*) represent residual chromatin.

PLATE I

Explanation of Figures

FIG. 1. Trophic ciliate showing typical nuclear apparatus, food inclusions, contractile vacuole and cytoplasmic vacuoles. Note the plaque-like arrangement of the macronuclear chromatin.

FIG. 2. Ciliate about to undergo encystment prior to reproduction. The macronuclear chromatin has become dispersed and flocculent.

FIG. 3. Early division cyst. Micronucleus enlarged and the macronuclear chromatin beginning to collect in aggregates.

FIGS. 4–8. Representative micronuclei during mitosis.

FIG. 9. Micronucleus in metaphase and macronuclear chromatin in the form of irregular aggregates.

FIG. 10. Later stage. Macronucleus elongating.

FIG. 11. Daughter micronuclei at the poles of the elongated macronucleus.

FIG. 12. Constriction of the macronucleus.

FIG. 13. Plasmotomy completed and the two daughter macronuclei rounded up. No indication of chromatin differentiation for elimination as yet.

FIG. 14. Budding off of extrusion chromatin (*).

FIG. 15. Slightly later stage.

FIG. 16. About the same condition as the previous stage and a timed preparation. This cyst represents the first one formed after emerging from the resistant cyst. Note small size.

FIG. 17. Probably a case of binary fission with the two daughter ciliates about to leave the wrinkled cyst membrane. Note extrusion chromatin (*).

FIG. 18. Prophase of the second division. The extrusion chromatin has been absorbed in the cytoplasm.

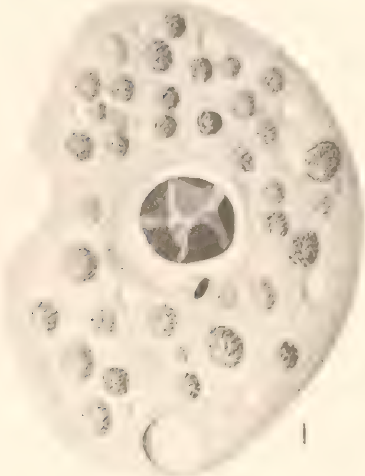
FIG. 19. Constriction of the macronuclei for the second division.

FIG. 20. Second cell division completed. Extrusion chromatin being given off from each daughter macronucleus.

FIG. 21. Four small ciliates about to emerge from the division cyst. Within the cytoplasm of each will be seen the residual ball of chromatin (*).

FIG. 22. A case of quadruple division without encystment. This represents the last stage with the daughter ciliates completely reorganized and about to separate.

FIG. 23. A free-swimming ciliate just after emerging from the division cyst. The residual ball of chromatin (*) has not been absorbed as yet.



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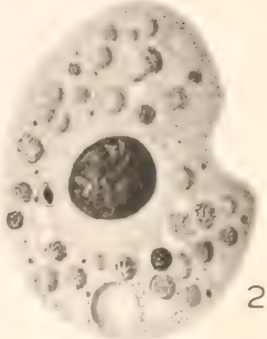

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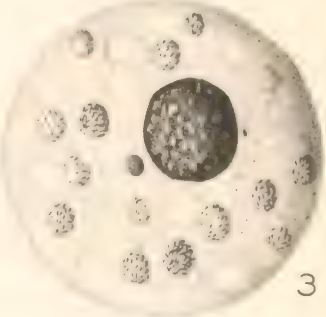

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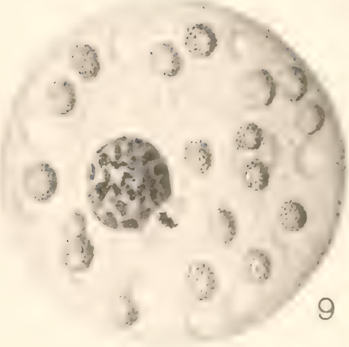

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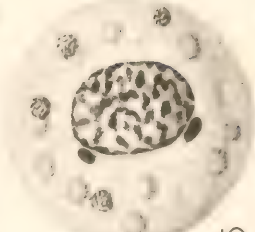

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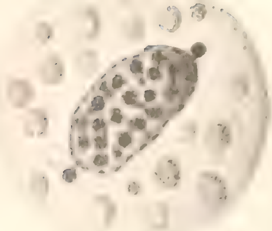

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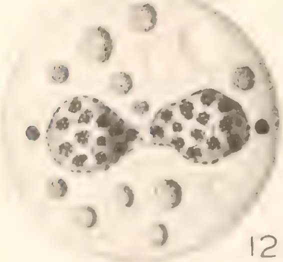

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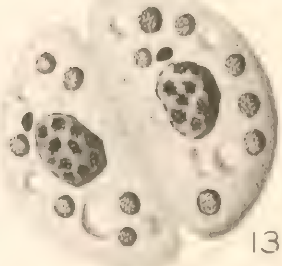

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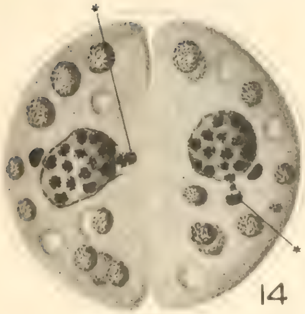

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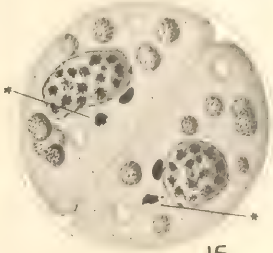

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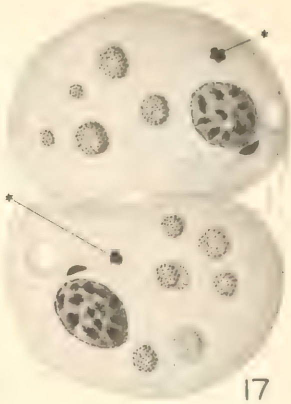

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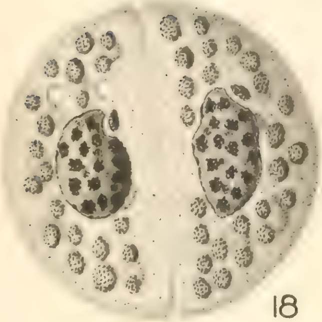

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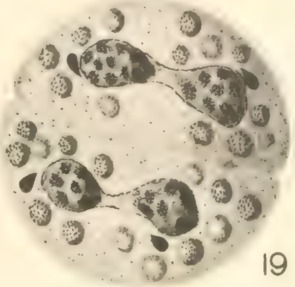

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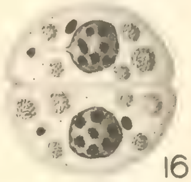

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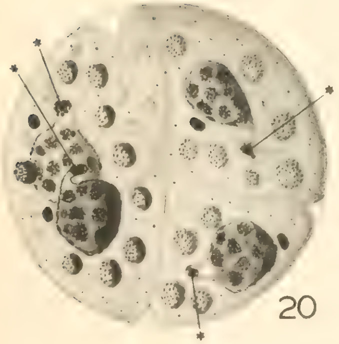

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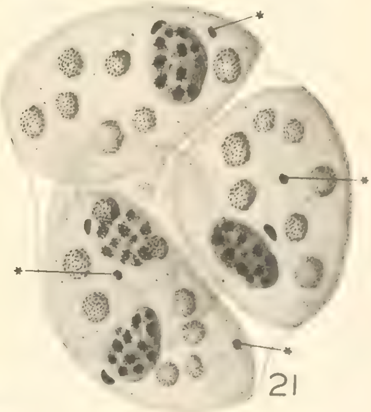

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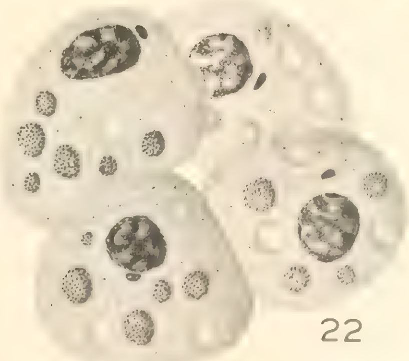

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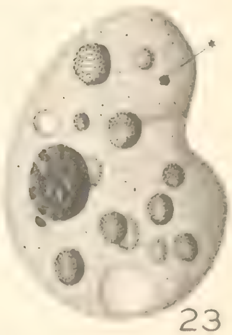

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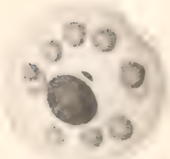
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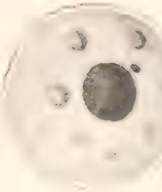
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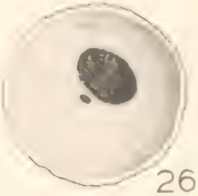
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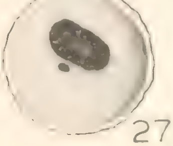
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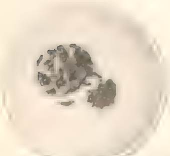
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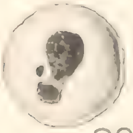
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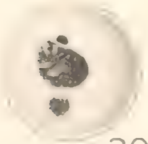
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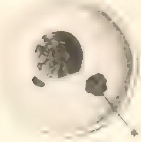

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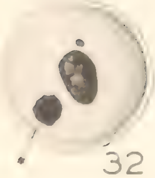

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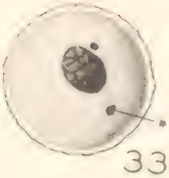

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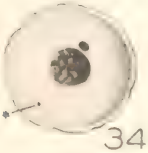

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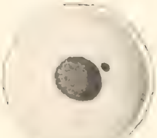
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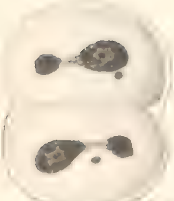
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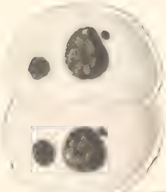
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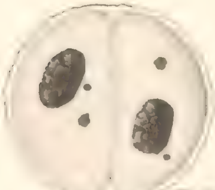
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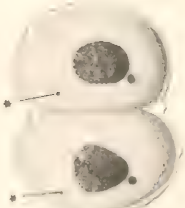

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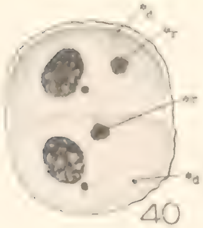

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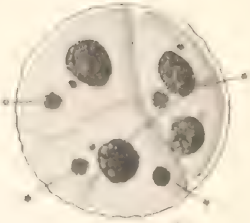

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pacted. This compacted region varies in size from one-third to one-half the whole nucleus (Plate II, Fig. 27). Very rapidly the compacted area buds off from the rest of the macronucleus leaving its chromatin in an irregular granular condition (Plate II, Fig. 28). The compact bud moves farther away from the nucleus until the connection between the two severs and the deeply-staining chromatin rounds up in the cytoplasm (Plate II, Figs. 29, 30 and 31). The amount of chromatin extruded varies considerably in the different cysts. In extreme cases it forms a ball as large as the remaining reorganized macronucleus but usually it is somewhat smaller (Plate II, Figs. 31 and 32). The ultimate resorption of the cast-out chromatin takes place by a gradual diminution in size but no apparent diminution in staining capacity (Plate II, Figs. 33 and 34). Within a few hours after encystment the extruded chromatin has disappeared and there is no further activity within the cyst until excystment. The refringent bodies have gradually disappeared during this process of nuclear reorganization and the resting cyst then possesses a very clear, slightly granular cytoplasm in which the micro- and macronucleus are embedded (Plate II, Fig. 35).

PLATE II

FIG. 24. Newly-formed resistant cyst characterized by the possession of food spheres.

FIG. 25. Later stage showing the diminution of food bodies and the concomitant appearance of the small, refringent bodies.

FIG. 26. The food bodies have entirely disappeared and the refringent bodies are numerous.

FIG. 27. Early stage in the differentiation of the extrusion chromatin within the macronucleus. The extrusion chromatin stains more intensely than the chromatin which is destined to remain.

FIGS. 28–30. The budding off of the ball of extrusion chromatin.

FIG. 31. The connection between the extrusion bud and the macronucleus has broken. Note the globular condition of the large extrusion mass (*).

FIG. 32. A large extrusion mass (*) somewhat later than the preceding stage.

FIGS. 33–34. The extrusion chromatin mass (*) diminishes in size. Note also that the refringent bodies within the cytoplasm have disappeared.

FIG. 35. A reorganized resistant cyst with compact clear cytoplasm and a smoothly granular, compact macronucleus. In this condition the resistant cyst will remain until the cultural conditions are altered sufficiently to induce its excystment.

FIGS. 36–39. Chromatin elimination from the macronuclei of two-cell resistant cysts duplicating, in each cell, the conditions seen in the single-celled resistant cyst.

FIG. 40. Two-celled resistant cyst in which cyst formation occurred before the extrusion chromatin of the divisional reorganization was completely absorbed. The divisional extrusion chromatin is represented by the small, deeply staining balls (*d) while the resistant cyst extrusion chromatin is represented by the large masses (*r).

FIG. 41. Four-cell resistant stage showing typical extrusion chromatin in each cell. This type of cyst is relatively rare.

FIG. 42. Small, clear, cyst-like structures which appeared in a few old liquid cultures and which are thought to represent degenerate resistant cysts.

We have found a few cases where two buds of waste chromatin have been extruded from the reorganizing macronucleus but these are rare.

When, as often happens, division cysts become resistant cysts there is a complete reorganization within each of the daughter ciliates identical with that of the single-celled cyst described above. Usually there appears to be enough time for the divisional reorganization of the macronucleus to proceed to completion and the subsequent resorption of the extrusion chromatin to take place before resistant cyst macronuclear reorganization sets in (Plate II, Figs. 36, 37, 38, 39 and 41). Rarely are there found resistant cysts where both divisional extrusion chromatin and resistant cyst extrusion chromatin are present (Plate II, Fig. 40). Also resistant cysts containing two cells are much more frequent in occurrence than those containing four cells.

Discussion

In our opinion the actuality of macronuclear reorganization involving the elimination of residual chromatin may possibly be demonstrated universally among the holotrichous ciliates. The definite establishment of the elimination of residual chromatin as a single ball during divisional reorganization has been made in the following forms: *Kidderia* (*Conchophthirius*) *mytili* (Kidder, 1933*a*), *Ancistruma isseli* (Kidder, 1933*b*), *Conchophthirius anodontæ*, *C. curtus*, *C. magna* (Kidder, 1934), *Myxophyllum* (*Conchophthirius*) *steenstrupii* (Rossolimo and Jakimowitsch, 1929), *Allosphærium convexa* (Kidder and Summers, 1935) and *Urocentrum turbo* (Kidder and Diller, 1934). A number of other species undoubtedly fall into this group if we can judge by the published reports (*see Loxocephalus*, Behrend, 1916 and *Eupoterion pernix*, MacLennan and Connell, 1931). Post-divisional chromatin elimination, i.e. the casting into the cytoplasm of residual chromatin from each of the daughter macronuclei after separation, is known to occur in *Ichthyophthirius multifiliis* (Haas, 1933), *Colpidium colpoda*, *C. campylum* and *Glaucoma scintillans* (Kidder and Diller, 1934), *Chilodonella labiata* and *C. faurei* (MacDougall, 1936).

In all the cases cited above the chromatin to be eliminated is differentiated within the macronucleus prior to its division and thus advertises itself. All species, therefore, in which no differentiation into regions occur prior to fission have been described as having a "clean" macronuclear division. As the vast majority of ciliates divide without encystment, and the two daughter cells separate immediately after fission, this phase of their cytology has been neglected. It seems entirely possible to us that if attention were paid to the young daughter cells after fission the occurrence of macronuclear reorganizational processes would be discovered in a great many if not all species. Another possibility that suggests itself is that the many cases of chromatin-like fragments in the cytoplasm so often reported in ciliate studies may be explained by some process of macronuclear elimination during reorganization. We wish to emphasize the necessity for more thorough and critical cytological work with this problem in mind to determine whether or not *macronuclear reorganization with chromatin elimination will be found to be a universal principle applicable to all holotrichous ciliates.*

As to the exact significance of this regular though complicated process, we are still unable to say. It has been suggested (Kidder, 1933*a*, 1933*b*, 1934; Kidder and Diller, 1934) that the eliminated chromatin represents worn-out material and the process might be a cleaning out of the macronucleus toward a perfect organization. It was further suggested (Kidder and Diller, 1934) that the profound reorganization which occurs at every division of *Colpidium colpoda*, *C. campylum* and *Glaucoma scintillans* might account for the fact that conjugation rarely occurs in these species, the reorganizational process serving to restore the cells to their fundamental condition and thereby decreasing the necessity for conjugation. The above suggestion seems to us to apply as well as any other to *Colpoda cucullus*.

Concerning the drastic reorganization that occurs immediately after the resistant cyst is formed, it seems logical to suppose that this represents the ridding of the macronucleus of materials no longer needed in the state of decreased or suspended metabolism. Materials accumulated in the macronucleus through the very activity of encystment may be detrimental to the resting cell or to the process of excystment, to come at a later date. Unfortunately we have very few records of what goes on within the resistant cysts of the various species of ciliates with which to make comparisons. We know from a few sources (see Tittler, 1935) that a process of endomyxis sometimes accompanies encystment, whereby the old macronucleus is completely discarded and a new one formed from micronuclear material. These cases would seem to represent simply a different method for accomplishing the same general result as occurs in *Colpoda cucullus*, the production of a purified macronucleus.

That the reorganizational process occurring within the resistant cyst takes the place of the divisional reorganization of the macronucleus is denied by direct observation. In the very first division after emergence from the resistant cyst the normal reorganizational chromatin elimination occurs. This was determined by timed preparations and Figure 16 illustrates a first division cyst. The small size is usual as the ciliates emerging from the resistant cysts are very small and usually reproduce before full growth is attained, a condition noted in the case of *Tillina magna* by Gregory (1909). The time factor may play an important rôle here, however, as there is the possibility of the aging of the macronucleus during its long period within the resistant cyst, resulting in the necessity for reorganization immediately upon again taking up an active life.

The actuality of a regular and predictable macronuclear reorganization with the elimination of quantities of chromatin during division and within the resistant cyst has been established for *Colpoda cucullus* but a completely satisfactory explanation of its significance awaits further investigation. Experiments are now under way which, it is hoped, will throw some light on this question.

SUMMARY

1. A complete description of the nuclear activity of *Colpoda cucullus* Muller is given for the first time.

2. In our strain the normal method of reproduction takes place within a thin cyst membrane. Usually two divisions result giving rise to four daughter organisms which break out of the cyst and repeat the process. Occasionally binary fission occurs within the cyst. Rarely quadruple division occurs without encystment, as described by Penn (1937).

3. Following each cell division there occurs a reorganizational process within the daughter macronuclei resulting in the elimination of a quantity of residual chromatin. The residual chromatin is cast into the cytoplasm where it is absorbed. Elimination of residual chromatin is regular and synchronous in each cell whether the division has occurred within a cyst or not.

4. When cultural conditions are poor resistant cysts are formed.

5. The resistant cysts are formed by the secretion of a heavy cyst membrane, the absorption of the food inclusions and the concentration of the whole protoplasmic mass.

6. Immediately following the formation of the resistant cyst membrane the macronucleus undergoes a profound reorganization during which a variable, but always a considerable amount of chromatin is budded off and cast into the cytoplasm where it is absorbed. No micronuclear activity occurs at this time.

7. The question of chromatin elimination from the macronuclei of holotrichous ciliates is discussed and the opinion expressed that this phenomenon may represent a universal principle.

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EMBRYONIC DETERMINATION IN THE ANNELID, SABELLARIA VULGARIS

I. The Differentiation of Ectoderm and Endoderm When Separated through Induced Exogastrulation

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Introduction

The method of transplantation has brought to light the abilities of certain embryonic parts to induce, or organize, the development of other embryonic structures in adjacent tissues. The use of this technique has, however, only recently been extended to the so-called "mosaic" eggs. Tung (1934) reported experiments involving the fusion of eggs at the two-cell stage, fusion of micromeres and macromeres, and rotation of micromeres in the eggs of *Ascidiella*. He found no deviation from the normal in the development of the cells giving rise to notochord and muscle. However, there was evidence that the sense organ and the ectodermal adhesive organ were induced. Two years later, there appeared abstracts by Hörstadius (1936) and by the writer (1936) describing the development of transplanted blastomeres of *Cerebratulus* and *Sabellaria* respectively. Hörstadius (full report, 1937) found no evidence of induction in fusions of various quartets of the 16-cell stage. The essentially similar results of the writer will be described in greater detail in the second paper of the present series.

It is the purpose of this first paper to describe the results of an experiment which, though similar to isolation experiments, possesses certain of the added advantages of the transplantation method. The experiment consists of the production of larvæ in which the endoderm cells grow out, during gastrulation, from their normal position underlying the ectoderm. This affords a way of testing the independent differentiation of the two layers and, by inference, the effect of one layer upon the other during normal development.

Material and Methods

The egg used is that of the marine annelid, *Sabellaria vulgaris*. That this is an egg of the classical mosaic type is shown by the completely partial development of isolated blastomeres (Hatt, 1932). The procedure for obtaining fertilized eggs of this species has been described elsewhere (Novikoff, 1937). The larvæ of this species are closely similar to those described by Wilson (1929) for the two European species, *alveolata* and *spinulosa*.

Exogastrulae can be produced in considerable numbers by a treatment which removes the vitelline membranes of fertilized ova. A variation of the method employed by Hatt (1931) has proved to be quite effective. At any time before they have begun to change their form in preparation for cleavage, the eggs are washed once with an isotonic NaCl solution brought to pH 9.6 by the addition of Na_2CO_3 (.98 gram in 1 liter of solution) and are then placed into 5 cc. of the same solution. The eggs adhere to each other in this medium, forming large aggregates, but as the egg membranes disappear, the aggregates break up. When this has occurred, 5 cc. of acidified NaCl solution (0.45 cc. 1.0 N HCl in 100 cc. 0.53 N NaCl) are added, bringing the medium to pH 8.2. When the eggs have settled to the bottom of the dish, they are washed once in sea water and transferred to a Syracuse dish containing fresh sea water. Among the swimming larvæ which develop, the exogastrulæ can readily be distinguished from the normal larvæ in the dish.

Representative stages in the development of exogastrulæ and control larvæ are described in the text. All text figures are camera lucida drawings of living larvæ; the description accompanying each figure refers not to that specific larva but is a general description of larvæ at that stage in development. The time intervals given are approximate, for temperatures ranging from 19° to 24° C.

Description of Results

It is possible to follow the details of ectodermal and endodermal differentiation in living larvæ. In the normal course of development the ectoderm gives rise to the following structures: a prototroch of long active cilia, at first encircling the animal but later incomplete on the dorsal surface; chromatophores, which are yellow when they form but later change to green; an apical tuft which disappears when replaced by short non-motile cilia; paired chætæ-sacs from which extend long serrated bristles; a single, short, non-motile posterior cilium; several long, non-motile dorsal cilia in the region of the prototrochal gap; a single orange-red eye spot on the left side, towards the dorsal surface; and rapidly moving cilia ("neurotroch") in a ventral depression leading to the mouth cavity of the fully formed trochophore. The endoderm differentiates into a tripartite gut consisting of œsophagus, stomach, and intestine. The œsophageal cells are lined internally with many long actively-moving cilia. The stomach and intestine are also ciliated but the motion of their cilia differs from that of the œsophagus; they move more slowly and remain more rigid during the effective stroke.

In order to compare the development of the eggs from which the membranes have been removed with that of the normal eggs, a series of stages in the development of both are described. Those stages have been chosen which show definite advances in structural differentiation.

The early cleavages of the membraneless eggs are similar in all respects to those of normal eggs. Although the details of the later cleavages have not been described for this species, any marked difference in this process between the two types of eggs would have been noted. But no such variations were apparent. The first noticeable departure from normal development appears at the time when the larvæ show the first signs of movement. This is usually between six and seven hours after fertilization and probably corresponds to the time when gastrulation is normally occurring.

Comparison of Complete Exogastrulæ with Normal Larvæ

Six to Seven-hour Larva.—Normal (Fig. 1*A*). There is no regularity in the shape of the larvae at this time; it may vary from a spherical to a roughly conical form. The vitelline membrane, retaining the many wrinkles characteristic of the earlier stages in development, is still far removed from the body of the larva. Through the membrane, there project a group of long cilia at the future anterior end of the larva, and many short, rapidly moving cilia which form a narrow girdle completely encircling the larva.[1] These cilia constitute the apical tuft and the prototroch respectively. The larva is rather opaque and it is with difficulty that the large internal cells can be distinguished. Directed towards the inner cells and situated slightly posterior to the prototroch is the blastoporal indentation.

Exogastrula (Fig. 1*B*). These larvæ are more uniformly regular than the normal larvæ of the same age. Not obscured by the vitelline membrane as they are in the normal larvæ, the cilia of the apical tuft and prototroch are more easily distinguished. The distance between the apical end and the prototroch (i.e., the pretrochal region) is the same as it is in the normal larva, but the post-trochal region is considerably lengthened by a posterior outgrowth of cells. No blastopore is present.

Thirteen-hour Larva.—Normal (Fig. 1*C*). The larva has now rounded out and there are only slight irregularities in the surface.

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[1] The cilia in the early larvæ may be brought out more clearly with darkfield illumination.

It has elongated somewhat in the antero-posterior direction and is just beginning to assume the typical trochophore appearance. The apical tuft is directed forward as the larva swims, rotating on its longitudinal axis. The prototroch, which formed a complete ring about the animal at the preceding stage, is interrupted by a short region with no cilia; this gap marks the dorsal surface of the trochophore. At the posterior, broader end there projects a fine non-motile cilium. The surface of the larva now has a general gold-yellow coloration when viewed by transmitted light. Through the outer ectoderm cells the "anlage" of the gut is discernible. From a crescent-shaped cavity at the apical end, a narrow canal leads through the gut cells to open on the surface at the posterior end. The blastopore, which has become progressively smaller up to the eleventh hour of development, has completely disappeared.

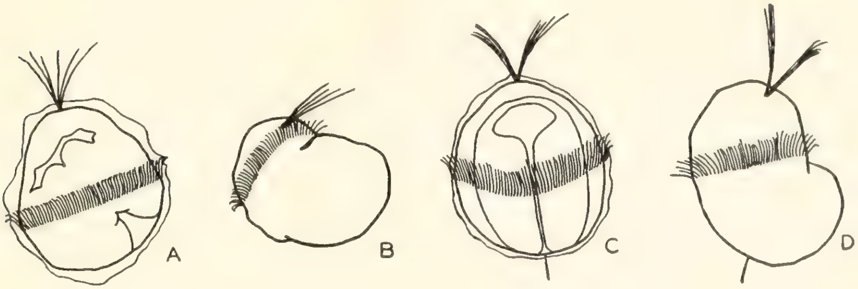

FIG. 1. *A*. Normal larva. Seven hours after fertilization. Shows apical tuft and prototroch. Posterior to the prototroch is the blastoporal indentation. *B*. Exogastrula. Seven hours. Viewed from posterior end. Shows apical tuft, prototroch, and elongate post-trochal region. *C*. Normal larva. Thirteen hours. Shows apical tuft, prototroch, posterior cilium, and differentiating gut. *D*. Exogastrula. Thirteen hours. Shows apical tuft, prototroch, posterior cilium, and elongate post-trochal region.

Exogastrula (Fig. 1*D*). This larva is considerably more elongate, antero-posteriorly, than the normal larva of the same age. The structure of the apical tuft, prototroch, and posterior cilium is normal, and the apical tuft is in the usual location. On the other hand, the relative positions of both the prototroch and the posterior cilium are strikingly different. The former is situated more anteriorly in the larva; the latter is to one side of the larva and projects laterally instead of posteriorly. The general yellow coloration of the embryo does not extend very far beyond the equator; the cells at the posterior end are colorless. The crescent-shaped cavity and the longitudinal canal present in the endoderm cells of the normal larva at this stage are not present.

Eighteen-hour Larva.—Normal (Figs. 2*A* and *B*). Since the preceding stage, the larva has become more spherical and more regular in outline. The membrane, almost free of irregularities, is more closely applied to the larval surface. The cilia of the apical tuft are now considerably longer and they move slowly in two distinct groups. The prototrochal gap has grown in size; the posterior cilium is still present, and the yellow pigment has become localized into a number of larger areas on the surface of the body. The endoderm cells within are now assuming the form of the gut; one can recognize the three main divisions—the ventral œsophagus runs anteriorly into the wider stomach, which in turn is continuous with the intestine. The only lumen visible in the gut is the narrow canal described in the earlier larva; it runs through the stomach and intestine and opens as the anus. Slightly anterior to the prototroch and on either side of the stomach there has developed a spherical structure, the chæta-sac, from which the long bristles later grow.

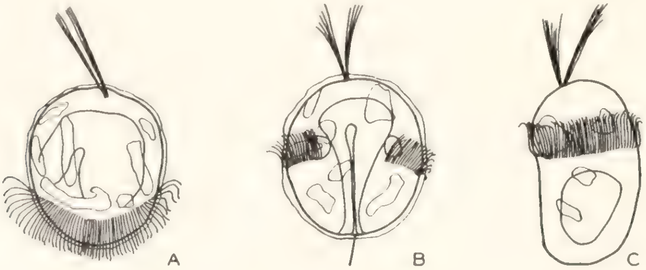

FIG. 2. Larvæ, eighteen hours after fertilization. *A*. Normal larva. Side view. Shows apical tuft, prototroch, gut, and pigmented areas. *B*. Normal larva. Dorsal view. Shows prototrochal gap, stomach and intestine portion of gut, and posterior cilium. *C*. Exogastrula. Post-trochal region is elongated and hollow.

Exogastrula (Fig. 2*C*). Changes similar to those described for the normal larva have occurred in the apical tuft, prototroch, and pigmentation of the exogastrula. The apical tuft is composed of two groups of long cilia. The prototrochal gap extends over the dorsal surface. The yellow pigment has become more localized, but since the pigment was originally restricted to the more anterior part of the larva, the posterior portion is completely colorless. The difference between this larva and the normal one of the same age is, however, much more pronounced than at the preceding stage. In contrast to the fairly spherical form of the normal, it is narrow and long. It possesses no internal gut and in the posterior half there appears a large cavity. The posterior cilium is again laterally displaced.

Twenty-four-hour Larva.—Normal (Figs. 3*A* and *B*). Important changes have occurred between this and the preceding stage of development. In the apical region there have appeared, alongside the apical tuft, a number of short, non-motile cilia. On the dorsal surface of the larva, in the region of the enlarged prototrochal gap, there are two or three long non-motile cilia. The posterior cilium is still short and non-motile. The large pigmented areas have been broken up into well-defined yellow chromatophores. The gut has acquired a continuous lumen, lined with rapidly moving cilia. The cilia of the œsophagus differ from those of both the stomach and intestine in that they are slightly longer, are more numerous, per unit surface area, and move more rapidly. In their movement, they are bent more than are the stomach-intestinal cilia. The œsophagus opens to the exterior through the mouth, the intestine through the anus. From each of the chætæ-sacs there now extend two bristles. The position and structure of these bristles are very characteristic. Arranged at intervals along their length are serrated "collars." As the larva swims about, the bristles, projecting posteriorly, are seen being rotated about the long axis of the animal. That muscles have developed can be deduced from the observed contraction of the larvæ and the movement of the chætæ-sacs and bristles.

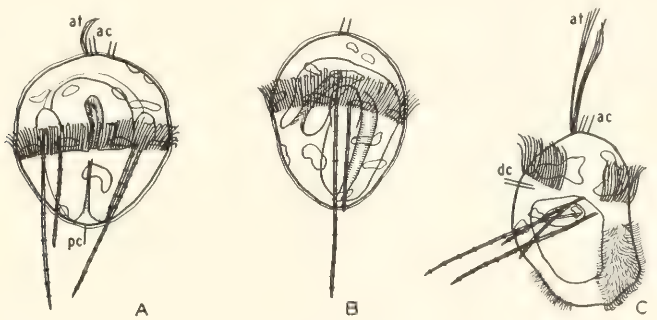

FIG. 3. Larvæ, twenty-four hours after fertilization. Abbreviations: *at*, apical tuft; *ac*, apical cilia; *pc*, posterior cilium; *dc*, dorsal cilia. *A*. Normal larva. Ventral view. Mouth, œsophagus, and intestine visible. The pigmented areas are broken up into well-defined chromatophores. Two pairs of bristles are present. *B*. Same larva as *A*. Side view. *C*. Exogastrula. Shows elongate, hollow, post-trochal region. On the surface, toward the posterior end, short cilia are visible.

Exogastrula (Fig. 3*C*). At this stage, the exogastrulæ can easily be distinguished, even with low magnification, from the normal animals. The most striking feature is the behaviour of the bristles when the animal is in motion. The larva rotates as usual as it moves in a forward direction. But the bristles project laterally instead of posteriorly, and they are accordingly turned over and over in a wide arc, rather than rotated on the longitudinal axis of the larva. Viewing the animal from above, the bristles therefore appear and disappear from sight. No gut is present, and the cavity of the preceding stage has increased noticeably in size. On the surface of the posterior portion of the larva there are now rapidly beating cilia, of the same size as the cilia in the gut of the normal larva. They are usually found in two distinct extensive areas, with the larger of the two areas covered with cilia characteristic of the normal œsophagus. It is possible to distinguish the single asymmetrically-placed posterior cilium. The larvæ show the following changes over the preceding stage, which are similar to the changes in normal development: (1) the appearance of short, non-motile cilia at the apical end; (2) the formation of smaller, yellow chromatophores, although in the exogastrulæ they are limited to little more than the anterior half of the larva; (3) the development of dorsal cilia near the gap in the prototroch; (4) the appearance of contractions of the larva, albeit these contractions are considerably weaker than those of the normal larva; (5) the formation of two pairs of bristles of the same notched structure as described for the normal.

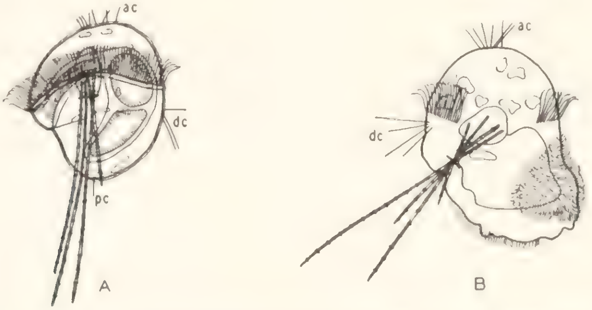

FIG. 4. Larvæ, twenty-nine hours after fertilization. Abbreviations as in Fig. 3. *A*. Normal larva. Side view. Mouth, stomach, and intestine are well-formed. The bristles on the left side only have been drawn. *B*. Exogastrula (slightly flattened). Dorsal view. Only four of the eight bristles have been drawn.

Twenty-nine-hour Larva.—Normal (Fig. 4*A*). The larva is beginning to assume the appearance of the fully-developed trochophore. The membrane is closely applied to the surface of the larva. The apical tuft has disappeared, and only the non-motile cilia remain at the anterior end. The prototroch is raised slightly, forming the so-called "hood fold" (Wilson, 1929). The single posterior cilium persists. The small yellow chromatophores are taking on a green tinge. The stiff dorsal cilia are longer and more numerous than in the previous stage. The œsophagus, lined with many active cilia, leads into the stomach. The stomach is partially separated from the intestine by an incomplete shelf; the intestine is lined with cilia of the type found in the stomach. The number of bristles varies from three to four pairs. On stimulation (i.e., when the slide or the dish is tapped gently), the larva rapidly contracts and the bristles are spread into a fan-like arrangement. After several seconds in this position, the animal returns to its usual form.

Exogastrula (Fig. 4*B*). The general appearance of the larva is similar to that of the preceding stage. The three or four pairs of bristles project laterally and, as the animal moves, are rotated in the manner described above. On stimulation, they are spread out as in the normal larva, although to a lesser extent. There is no internal gut, and a large cavity is present inside the larva. The chætæ-sacs project into the cavity. As in the normal larva of this age, only the apical cilia persist at the anterior end. The dorsal cilia have elongated and increased in number. The chromatophores, limited to about the anterior half of the larva, are becoming greener in color. The cilia on the exterior of the post-trochal region are found in two regions. Of these only the ventral region has the cilia of the œsophageal type.

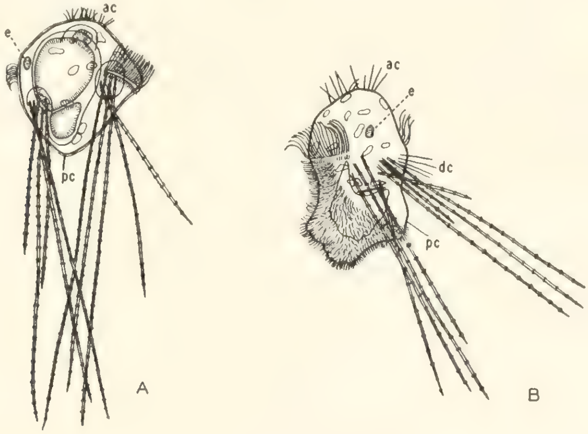

FIG. 5. Larvæ, forty-eight hours after fertilization. Abbreviations as in Fig. 3. *A*. Normal larva. Dorsal view. To the left of the stomach is the eye spot (*e*). *B*. Exogastrula. Dorsal view. A large part of the post-trochal region is covered with short cilia.

Forty-eight-hour Larva.—Normal (Fig. 5*A*). This is a well-developed trochophore. The post-trochal region has lengthened somewhat. The apical cilia are numerous and the number of dorsal cilia has also increased. There is a single posterior cilium. A second, posterior row of short cilia has appeared on the hood fold, just posterior to the long prototrochal cilia. On either side of the mouth there has developed a fold covered externally with cilia, the lip fold. The chromatophores have a more pronounced green coloration. On the left side of the larva, near the stomach, there has appeared a single eye spot, composed of closely packed orange-red granules. The gut is clearly differentiated into œsophagus, stomach, and intestine. The stomach has increased in size and has become more spherical. Running posteriorly from the mouth, in a groove along the ventral surface, are the long cilia which constitute the neurotroch. A larva at this time ordinarily has four pairs of bristles, which project posteriorly.

Exogastrula (Fig. 5*B*). Changes similar to those occurring in the normal larvæ have taken place between this and the preceding stage. The apical cilia have become more numerous. The prototroch is better developed and a row of short cilia has been added posterior to it. The dorsal cilia have increased in number and size, and the posterior cilium is single and dorsal. On the dorsal surface, anterior to the prototroch, may be seen the eye spot. Four pair of bristles project laterally as do the bristles of the earlier larvæ. There is no internal gut. The cilia characteristic of the œsophagus and the intestine, together with the cilia of the neurotroch and the lip fold, cover the post-trochal region almost completely. Special mention must be made of the eye spot, since it was not seen in all the larvæ examined. It could not be found in thirteen of the ninety-three larvæ studied.

Partial Exogastrulæ

There are always found in the culture dishes containing the membraneless larvæ a few larvæ which are intermediate in structure between those described as complete exogastrulæ and the normal. These larvæ are illuminating for an understanding of the structure of the more extreme type. Figure 6 shows two views of a well-developed trochophore of this type. Not all the structures are visible in the drawings, but the larva possesses every structure found in the normal animals, although these structures have in some cases differentiated in abnormal positions. The most outstanding feature of these larvæ is the gut structure. The stomach and intestine are clearly normal in form, position, and type of ciliation. The intestine opens posteriorly, as usual, through the anus. At the anterior end of the stomach there is a circular opening lined with cilia which move in a manner characteristic of the cilia of the œsophageal-stomach opening in the normal larva. But this opening leads here not to the œsophagus, but to the outer surface of the larva. In fact, there is no internal œsophagus at all. On the ventral surface of the larva is a long, conical outgrowth, completely covered with long actively-moving cilia which are characteristic of the œsophagus.

Discussion of Results

There are two important points of difference between larvæ which develop from eggs without membranes and normal larvæ. The first is the complete absence of an internal gut and the second the striking deviation from the normal form of the larva. Instead of possessing the fairly spherical shape, they have elongate bodies in which the posterior structures, the posterior cilium and the paired bristles, are dorsally displaced. The distortion of the posterior part of the larva may be accounted for if we assume that the endoderm cells, which differentiate normally inside the ectoderm to form the gut, grow out so that they come to lie on the surface of the larva.

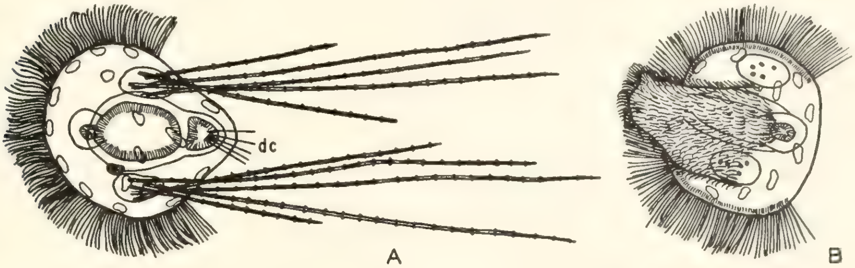

Fig. 6. Partial exogastrulæ. Seventy-two hours after fertilization. *A*. Dorsal view. The opening to the stomach, the stomach, and the intestine are visible. *B*. Ventral view. The bristles have been omitted in the drawing; only their points of origin in the chætæ-sacs are indicated. The opening to the stomach may be seen at the base of the long conical projection covered with short cilia.

That this posterior outgrowth of the endoderm cells has indeed occurred is shown by the following considerations: 1. The gut is completely lacking in these larvæ, and within the post-trochal region of the larva there is a large cavity. 2. The cells situated at the posterior end of these larvæ are not pigmented and never develop chromatophores. In the normal larva the chromatophores are found in the ectoderm; only the endoderm is colorless. 3. In the normal larva, motile cilia do not appear on the external surface of the post-trochal region until approximately thirty-five hours after fertilization. When they do develop, they are restricted to a narrow region, the neurotroch. But in the membraneless larva cilia begin to appear on the surface of the post-trochal region before the end of the first day of development. By twenty-four hours after fertilization they have covered two wide areas. That these cilia are not those of the neurotroch is clear from the fact that they cover two extensive regions and that they develop twelve hours earlier than do the neurotrochal cilia. Their appearance coincides precisely with the development, normally, of cilia on the inner surface of the endoderm cells. It is also possible to detect differences in the cilia which correspond to the differences between the œsophageal cilia and the cilia of the stomach and intestine.

We may therefore conclude that in these eggs the endoderm has been turned inside out. Whatever the mechanism involved, the end result of this process is comparable to the exogastrulæ that have been described in the sea-urchin (Herbst, 1893; Driesch, 1893) and in the amphibia (Holtfreter, 1933*a*, *b*).

In the partial exogastrulæ, the stomach and intestine develop normally, and only the œsophagus is exogastrulated. And, as we would expect on the basis of our assumption, the general form of the larva is more like the normal than is that of the complete exogastrula.

The most significant feature of exogastrula development in *Sabellaria* is the complete self-differentiation of both endoderm and ectoderm. In the amphibian egg, Holtfreter found that normal differentiation of the ectoderm is dependent upon contact with the mesendoderm. When the latter, instead of coming to lie beneath the ectoderm as it does normally, evaginates, it leaves the ectoderm a wrinkled, hollow sac in which no trace of differentiation into nervous tissue appears. On the other hand, the mesendoderm, although turned inside-out, undergoes self-differentiation; it produces gut, thyroid, pancreas, liver, notochord, musculature, kidney, and gonad. Thus, in the normal course of development, the endoderm differentiates independently of the ectoderm, but the ectoderm must be in contact with the endoderm, or gut roof, to differentiate normally. It is this gut roof which acts in the capacity of an organizer—which induces the formation of a nervous system in the ectoderm.

In *Sabellaria*, on the other hand, there is no deviation from normal differentiation of either the ectoderm or endoderm in the exogastrulæ. The endoderm cells retain their morphological polarity, so that they develop cilia on what is in these larvæ their external surface. And although the tripartite nature of the gut is lost, the type of cilia developed by the exogastrulated œsophagus cells differs from that of the stomach and intestine in the same way as do the normal œsophageal cilia from the normal stomach-intestinal cilia. The ectoderm, too, differentiates normally. The pigment develops and concentrates into yellow areas, which later break up into smaller chromatophores and become distinctly green in color. The apical cilia form at the anterior end and the apical tuft disappears. The serrated bristles develop normally, although the cells from which they arise have been displaced to one side (dorsally). On the dorsal side there develops the orange-red eye spot. Muscles, as evidenced by contractions of the larva, are also differentiated.

Thus, we find no evidence of any inducing effect of the endoderm upon the ectoderm, or vice versa, in the development of *Sabellaria*. There may possibly be some question concerning the self-differentiation of the eye spot in the exogastrula since in 13 out of 93 cases no eye spot was observed. In more than one hundred normal control larvæ studied, none was found without the eye spot. However, when individual untreated eggs are isolated in small drops of sea water beneath cover-slips it is found that they develop into larvæ in which only the eye spot fails to develop. In these isolates, it is clear that the absence of the eye spot is not due to the morphological absence of an inducing region below the ectoderm. The failure of the ectoderm to form the red pigment may perhaps be due to some general factor such as increased pressure or lower oxygen tension. The same is probably true of those exogastrulæ in which no eye spot is seen.

Thus, the absence of the eye spot in some of the exogastrulæ does not constitute a serious objection to the conclusion that there is no inducing ability of either the ectoderm or endoderm, as tested by their effects upon one another during development. It is possible that in the exogastrulæ the ectoderm has not been completely isolated from the endoderm. The cells which will give rise to the two layers are in contact with each other at one point. Through the study of exogastrulæ alone one can not exclude the possibility of diffusion of materials from one part of one layer across cell boundaries to any part of the other layer; nor can it be denied that the cells may exercise inducing effects upon each other during cleavage. These criticisms merely point to the limitations of this mode of approach. To overcome them, another method may be used—that of transplantation of blastomeres. The results of such experiments in the egg of *Sabellaria vulgaris* form the subject-matter of the second paper in this series.

SUMMARY

1. A simple method for the production of complete and partial exogastrulæ in *Sabellaria vulgaris* is described.

2. In the complete exogastrulæ, no internal gut is formed. In the partial exogastrulæ there is no internal œsophagus, but the stomach and intestine develop normally.

3. Both the ectoderm and endoderm show complete self-differentiation in the exogastrulæ, indicating that one layer exercises no inducing

effect upon the other between six and forty-eight hours of development, from gastrulation to the well-developed trochophore.

It is with pleasure that the writer expresses his gratitude to Professor L. G. Barth for untiring assistance and constant encouragement throughout the course of this investigation.

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EMBRYONIC DETERMINATION IN THE ANNELID, SABELLARIA VULGARIS

II. Transplantation of Polar Lobes and Blastomeres as a Test of their Inducing Capacities

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Introduction

E. B. Wilson (1904*a*) demonstrated that the egg of the annelid, *Lanice*, belonged to the group of so-called mosaic eggs, since isolated halves of the two-cell stage developed into partial embryos. The anterior cell produced an embryo which lacked the post-trochal region; in the embryo formed from the posterior cell there was present a nearly typical post-trochal region. Delage (1899) had previously described a dwarf embryo from an egg-fragment of the same species. That annelid eggs, generally, are of the mosaic type is shown by the experiments of Penners (1924, 1926) with *Tubifex*, of Tyler (1930) with *Chætopterus*, and of Hatt (1932) with *Sabellaria*.

Wilson (1929) summarizes the evidence which indicates that there is no fundamental distinction between the mosaic and regulative types of ova. Among regulative eggs, where correlative differentiation, or embryonic induction, is most prominent, mosaic features can be found, and in mosaic eggs, there are suggestions that embryonic induction may play a part in early development. Wilson suggests that the polar lobe of such eggs as *Dentalium* may function as an organizing region similar to the dorsal blastoporal lip of amphibia, since only when the lobe is present does the larva develop the apical tuft and the post-trochal region. However, in the absence of transplantation experiments, no final conclusions could be reached.

Schleip (1929) describes a "natural experiment" in which a second polar lobe is added to the egg of *Dentalium*. Among giant eggs, some are found which appear to be fusions of two ova at their vegetal hemispheres. In these eggs, a single large polar lobe may be formed, which goes in its entirety into one of the cells. This leaves one egg with no lobe, and the other with two. However, such eggs do not develop. Schleip then tried to transplant isolated polar lobes to blastomeres, but all attempts were unsuccessful.

Transplantation experiments can readily be performed in the egg of *Sabellaria vulgaris.* In this species, large polar lobes, similar to those found in *Dentalium,* are formed in the course of the first three cleavages. Although the egg is only about sixty micra in diameter, it is not difficult to remove the polar lobe or to separate individual blastomeres. Both blastomeres and polar lobes can be fused together in desired combinations and the eggs reared through a well-developed trochophore stage. In all, 247 successful transplant operations have been studied, including 80 in which either the first or second polar lobe was transplanted.

MATERIAL AND METHOD

All experiments reported in this paper were performed at the Marine Biological Laboratory, Woods Hole, Mass., during the summers of 1935, 1936, and 1937. The animals used were dredged from Vineyard Sound and the eggs were obtained in the manner described elsewhere by the writer (1937). The egg of *Sabellaria vulgaris* possesses a tough, wrinkled vitelline membrane which resists cutting with glass needles. In addition, within the membrane, in the perivitelline space, there is a dense jelly which makes difficult the separation of individual blastomeres and which completely prevents bringing blastomeres together. Thus, to perform transplantations, it is necessary to first remove both membrane and jelly from the egg.

The vitelline membrane is removed from fertilized eggs of *Sabellaria* by treatment with an isotonic solution of NaCl, brought to pH 9.6 by the addition of Na_2CO_3 (Novikoff, 1938). In most cases, the eggs are thus treated within the ten minutes that elapse between the formation of the first polar body and the second. After they have been washed once in sea water, the denuded eggs are placed into a Syracuse dish of freshly-filtered sea water. The dish is allowed to remain without disturbance on the stage of a dissecting microscope. Within two or three minutes, the eggs have settled and are adhering to the glass bottom of the dish. By means of fine glass needles, each egg is then lifted from the jelly which remains adherent to the bottom. This process may have to be repeated several times in order to remove the jelly completely. The jelly being invisible, its removal can best be ascertained by bringing together the individual eggs; they come into contact with each other only when the jelly has been removed. At this time the eggs are quite sticky and if allowed to adhere too long to the dish they flatten out. Since such flattened eggs do not develop normally, it is important that the eggs be lifted, at close intervals, from the bottom of the dish.

The eggs are cut, free-hand, under the dissecting microscope. In order to determine the orientations of cells when fused, small spots are marked on the eggs before they are cut. This is done by bringing into contact with the surface of the egg the open end of a fine capillary tube filled with agar, in which is dissolved a vital dye such as Nile Blue sulphate. In order to fill an exceedingly fine bore, the following procedure is followed. A short piece of capillary tubing is partly filled by immersing one end in a warm solution of the agar containing the dye. When the agar has cooled, that part of the tube which has no agar is heated by a microflame and pulled out to the desired width of bore. The other end of the tube is then sealed off and the microflame applied to the part of the agar nearer the narrowed end. The agar melts and moves into the free end of the tube. On cooling, the agar, in many cases, remains at the opening of the tube. The tube is brought into contact with the egg by means of a Zeiss- Peterfi micromanipulator. The length of time during which the agar must remain in contact with the egg varies with the concentration of dye used. In some cases, where it is possible to determine the polarity of the isolated cell without previously staining a particular region, the entire cell is stained before transplantation. This is usually done before the membrane is removed, and a dilute solution of Nile Blue sulphate in sea water is used.

To effect the fusion of blastomeres it is only necessary to bring them into contact with each other, after the membrane and jelly have been removed. It is usually sufficient to press them together for several seconds, although this may, in some cases, have to be repeated several times before they finally stay together.

Following the operation, the eggs generally develop into swimming larvæ within five to six hours after fertilization. They can at that time no longer be left in the open dish for they soon swim to the surface, where they are quickly torn by the surface film. The procedure which leaves the least amount of surface exposed to the air and which, at the same time, is most convenient for the detailed study of the living larvæ, is to allow the embryos to develop in small drops of sea water between a glass slide and coverslip. When an embryo begins to show signs of movement, it is transferred, by means of a mouth pipette (Hörstadius, 1937*a*), to a small drop of sea water. Evaporation from the drop is prevented by sealing the edges of the coverslip with a thin layer of vaseline.

The results to be described are based on a total of 403 experimental larvæ, 156 developing from isolated blastomeres and 247 from fusions of various blastomeres. The percentages of larvæ surviving are: 73

per cent on the first day, and 51 per cent on the second day, from isolated cells; 91 per cent on the first day, and 74 per cent on the second day, from combinations of blastomeres. As described in the first paper of the present series (Novikoff, 1938), a great number of the larvæ arising from denuded eggs develop into exogastrulæ, in which there is no internal gut and the endoderm cells are turned inside out. Under the coverslips, all structures differentiate in the larvæ, just as they would in larger volumes of sea water, with the exception of the orange-red eye spot, and, to some extent, the yellow-green chromatophores. These are frequently absent or irregular. That the failure of the eye spot and the chromatophores to develop normally is due to some general factor such as lowered oxygen tension or increased pressure, is indicated by the development of normal eggs (i.e., eggs from which the membranes have not been removed) under similar conditions. Although the cell arrangement and the tissues of the larvæ from such eggs are manifestly similar to those of normal larvæ, the eye spot does not form and the chromatophores show the same variable character as do those of the membraneless larvæ.

Normal Development

Changes in the egg of *Sabellaria vulgaris* at maturation and during fertilization are described elsewhere (Novikoff, in press). Both polar bodies are formed after insemination and cleavage begins approximately twenty minutes[1] after the extrusion of the second polar body.

In the course of the first cleavage division, a large part of the vegetal hemisphere of the egg becomes constricted in the form of a spherical lobe. Figure 1, *A* is a photograph of an egg at the "trefoil" stage, when the polar lobe is at its maximum size. When viewed from the side, the lobe appears to be equal in size to either of the first two blastomeres. But when viewed from the vegetal pole, it is seen to be considerably smaller. The visible constituents of the lobe cytoplasm do not differ from that of either blastomere, except that there is no spindle area in the lobe. About fifteen minutes after it first appears, the polar lobe flows into one of the blastomeres. This blastomere is the *CD* cell and it is now much larger than the other, the *AB* blastomere (Fig. 1, *B*). Figure 1, *C* shows an egg during the second cleavage, when the second polar lobe is at its maximum size. This lobe forms in the *CD* blastomere only, and is smaller than the first lobe. When it flows back into one of the daughter cells at the completion of the division, the four quarter-blastomeres consist of two equal-sized cells, *A* and *B* (the products of the division of *AB*), a slightly larger cell, *C*, and a much larger cell, *D* (Fig. 1, *D*). It is the *D* blastomere which has received the contents of the second polar lobe. During the next division, when the micromeres are produced, a third polar lobe, formed from the *D* cell, flows into the *D* macromere, 1*D*. This lobe is smaller than the second lobe and is more variable than the preceding lobes;

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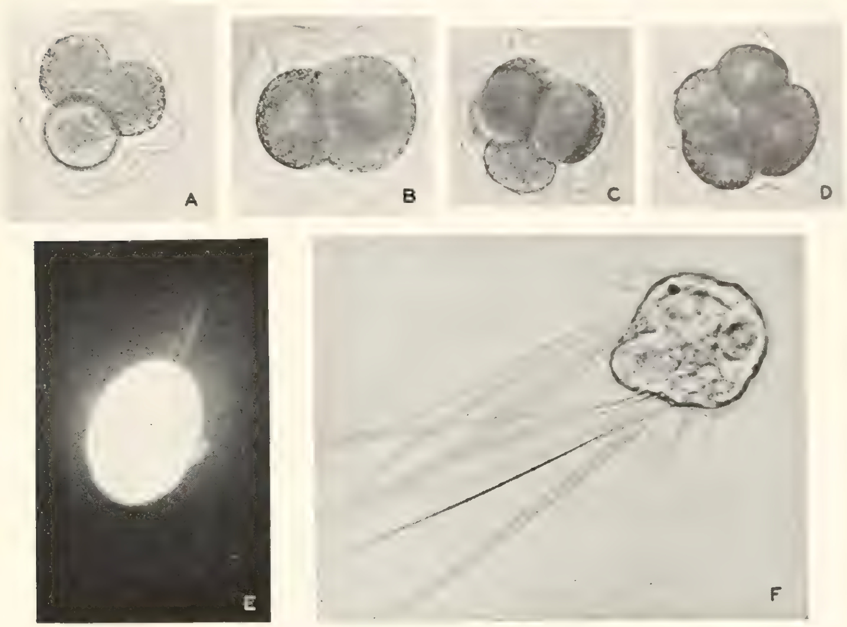
[1] All time intervals are for room temperatures, varying from 19° to 25° C.



FIGS. 1–10. All figures, except 1 and 5, are composed of camera lucida drawings of living larvæ, magnified approximately 260 times. Abbreviations used are:

p	prototroch
at	apical tuft
ac	apical cilia
ec	cilia of exogastrulated endoderm cells
pc	posterior cilium
dc	dorsal cilium
pb	post-trochal bristle
e	eye
m	mouth

FIG. 1. Photomicrographs of living eggs of *Sabellaria vulgaris*. *A*. Trefoil stage. Side view. The polar lobe is slightly out of focus. *B*. Two-cell stage. Blastomere *CD* is to the right of *AB*. *C*. Second cleavage. The second polar lobe and two of the quarter-blastomeres are in sharp focus; the other two cells are not in focus. *D*. Four-cell stage. Seen from the vegetal pole. The large *D* cell is to the right and the *A* cell is uppermost. *E*. Early trochophore larva. Sixteen hours after fertilization. Photographed with dark-field illumination; shows apical tuft and prototrochal cilia. *F*. Later trochophore larva. Forty-eight hours after fertilization; shows stomach, intestine, dark eye spot, prototrochal cilia, and post-trochal bristles.

in many cases this lobe does not become distinctly separated from the dividing *D* cell. The later cleavages of the egg have not been described.

A detailed description of the development of the larva is presented in the first paper of this series (Novikoff, 1938). In the normal course of development, the ectoderm gives rise to the following structures: a prototroch of long active cilia; chromatophores; an apical tuft; non-motile apical cilia; paired chætæ-sacs from which extend long, serrated bristles; a single posterior non-motile cilium; several long non-motile cilia on the dorsal surface; an eye spot; and a neurotroch of rapidly moving cilia. The endoderm differentiates into a tripartite gut consisting of œsophagus, stomach, and intestine. In many of the membraneless larvæ, the gut is exogastrulate and the three portions can not be distinguished. Of the ectodermal structures, those most easily observed in living embryos—and therefore the ones best suited for the present study—are the apical tuft, the post-trochal bristles, the prototrochal cilia, and the apical cilia. The apical tuft forms at about six hours after fertilization, and persists for approximately twenty hours (Fig. 1, *E*). Before it disappears, there develop at the apical end, a number of stiff cilia; these apical cilia remain throughout larval development. The prototrochal cilia appear at about the same time as the apical tuft and they remain throughout larval development. The post-trochal bristles make their appearance toward the end of the first day of development. They increase in length and number as development progresses (Fig. 1, *F*).

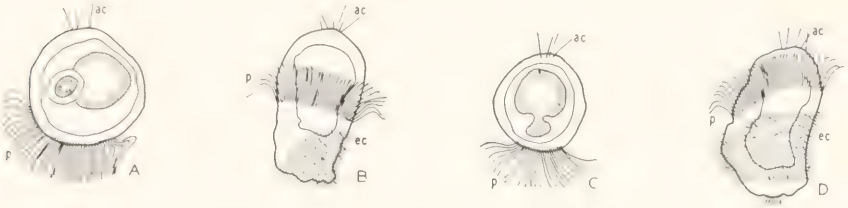


FIG. 2. Larvæ from isolated blastomeres. *A*. *E-PL1* larva, seventy-one hours; with internal gut. *B*. *E-PL1* larva, fifty hours; with exogastrulated endoderm. *C*. *AB* larva, fifty-one hours; with internal gut. *D*. *AB* larva, fifty-six hours; with exogastrulated endoderm.

ISOLATION EXPERIMENTS

E-PL1.—An egg from which the polar lobe is removed at the trefoil stage is labelled *E-PL1*. The first cleavage of *E-PL1* differs from the normal second cleavage in that no polar lobe is formed. As a result, the quarter-blastomeres are equal in size. In addition, no lobe is formed at the next division. The larvæ of such eggs differ from the normal larva in that: (1) no apical tuft forms; (2) no post-trochal region (including the bristles) appears; and (3) the prototrochal cilia, although of the same size as in normal larvæ, are at the posterior end of the larval ectoderm (Fig. 2*A*, *B*). Although no apical tuft is present, at approximately twenty-four hours after fertilization the typical non-motile cilia appear.

AB.—The isolated *AB* cleaves without the formation of polar lobes and gives rise to a spherical larva similar to that of *E-PL1*. It lacks the apical tuft and post-trochal bristles. It develops normal prototrochal and apical cilia, although the former are situated at the posterior end of the larval ectoderm (Fig. 2*C*, *D*).

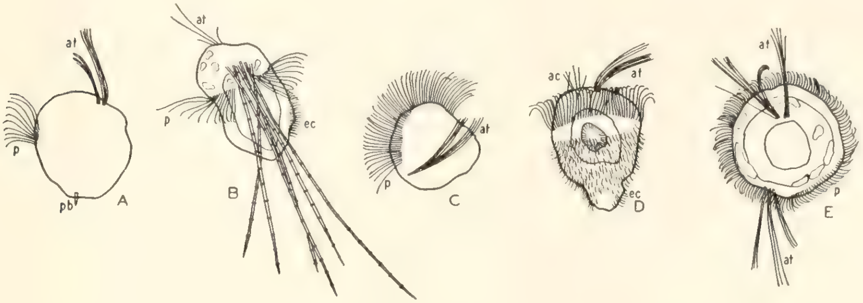


FIG. 3. Larvæ from isolated blastomeres. *A*. *CD* larva, twenty-five hours. *B*. *CD* larva, eighty-five hours. *C*. *CD-PL2* larva, fifty-six hours. *D*. *E-PL2* larva, forty-five hours. (On the next day, the apical tuft was no longer present.) *E*. *E-PL2* larva, eighteen hours. It is exceptional in that it possesses two apical tufts.

CD.—During the first cleavage of *CD* there is formed a polar lobe of the same size as the normal second lobe. After the division, it flows into the *D* cell. At the second cleavage, another, smaller lobe forms from the *D* cell. This lobe has the variable character of the normal third lobe. The early *CD* larvæ appear to be quite normal—the prototrochal cilia are in their usual position and the typical apical tufts are formed (Fig. 3, *A*). However, since the cilia and tuft are of the normal size, they are, proportionately, too large for these reduced larvæ. Later, the paired bristles are formed. However, no apical cilia develop, and in many instances (17 out of 29), the apical tuft does not disappear when it does in controls (Fig. 3, *B*).

CD-PL2.—If during the course of the first cleavage of the *CD* blastomere, the polar lobe is removed, the next cleavage occurs without the formation of a lobe. The *CD-PL2* larva possesses a typical apical tuft but lacks the post-trochal structures as well as the apical cilia (Fig. 3, *C*).

E-PL2.—It is with difficulty that the second polar lobe is removed from a whole egg. In each of the three larvæ obtained, normal apical tufts and prototrochal cilia develop. (One larva possesses *two* apical tufts—Fig. 3, *E*.) The older larvæ show no post-trochal structures, but do develop apical cilia (Fig. 3, *D*).

BC.—Before the cells have shifted their position after the second cleavage, it is possible to divide them so that *BC* is separated from *AD*. The *BC* combination forms no polar lobe and gives rise to a larva which possesses the typical apical tuft and prototrochal cilia, and a few apical cilia. It lacks completely the post-trochal region (Fig. 4, *B*).

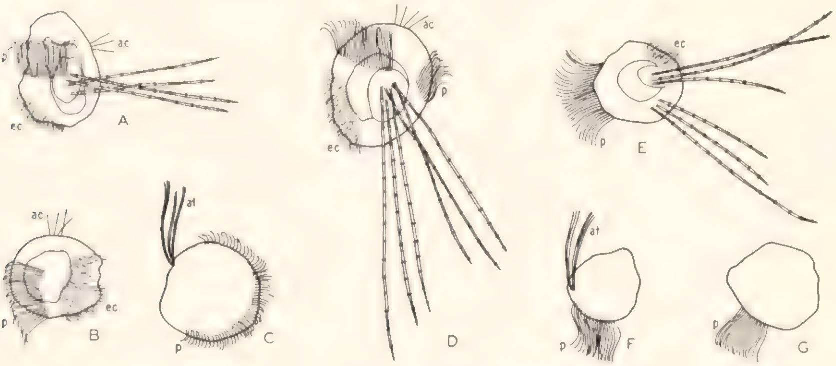


FIG. 4. Larvæ from isolated blastomeres. *A*. *AD* larva, forty-nine hours. *B*. *BC* larva, fifty hours. *C*. *ABC* larva, twenty-six hours. *D*. *ABD* larva, sixty-six hours. *E*. *D* larva, forty-seven hours. *F*. *C* larva, thirty-three hours. *G*. *D-PL3* larva, forty-one hours.

AD.—At the first cleavage of *AD*, a small lobe, which has the variable character of the normal third lobe, forms in the *D* cell. The larva developing from this combination of cells forms no apical tuft. It possesses normal prototrochal cilia and the typical post-trochal region from which extend the bristles. Apical cilia are also present (Fig. 4, *A*).

ABC.—By destroying the *D* cell at the completion of the second cleavage, *ABC* combinations are obtained. No polar lobe is formed at the first cleavage. The larva which develops possesses the typical apical tuft and apical cilia. However, no post-trochal bristles are produced (Fig. 4, *C*).

ABD.—Destruction of the *C* cell leaves the *ABD* blastomeres. At the next cleavage, the *D* cell forms a small polar lobe. The *ABD* larvæ develop normal prototrochal cilia, but no apical tufts. Later in development, apical cilia and post-trochal bristles appear (Fig. 4, *D*).

C.—No polar lobes are formed during the cleavage of the *C* blastomere. The larva developing from the *C* cell develops typical prototrochal cilia and apical tuft. But neither post-trochal structures nor apical cilia are formed (Fig. 4, *F*).

D.—During the first cleavage of the *D* cell, the small variable lobe appears and passes into the macromere, 1*D*. The *D* larva has typical prototrochal cilia and post-trochal bristles. But it develops no apical tuft and no apical cilia (Fig. 4, *E*).

D-PL3.—In some cases, the lobe formed by the *D* cell constricts sufficiently so that it may be removed. The larva developing from the *D-PL3* lacks the post-trochal structures as well as the apical tuft and apical cilia (Fig. 4, *G*).

TABLE I

Summary of larvæ obtained from isolation experiments.

	Number Operated	Number Surviving	Apical Tufts			Post-trochal Bristles		Prototrochal Cilia			Apical Cilia		
			+	−	?	+	−	+	−	?	+	−	?
E-PL1	30	21	0	21		0	11	21	0		14	0	
AB	36	25	0	24		0	18	23	0	1	11	6	1
CD	56	47	37	1	9	32	1	42	0	5	1	26	6
CD-PL2	8	5	5	0		0	4	5	0		0	3	1
E-PL2	3	3	3	0		0	2	3	0		2	0	
BC	10	8	6	2		1	5	8	0		6	0	
AD	6	3	0	3		3	0	3	0		2	0	1
ABC	10	8	7	0	1	0	5	8	0		4	1	
ABD	7	5	0	5		4	0	5	0		4	0	
C	17	11	7	3	1	0	7	11	0		0	7	
D	23	15	0	12	3	9	4	15	0		0	9	4
D-PL3	7	5	0	5		0	3	5	0		0	3	
	213	156											

Table I summarizes the isolation experiments. All combinations of cells develop prototrochal cilia. But only those combinations which include the substance of the *C* cell (earlier found in the first polar lobe) develop apical tufts; only those which have the 1*D* cell materials (and previously found in the three polar lobes) form the post-trochal region; and the apical cilia develop only when the *A* or *B* cell is present. Three exceptions, a *BC* larva with bristles, a *CD* larva with apical cilia, and an *E-PL2* larva with two apical tufts, will be discussed later.

Behavior of Isolated Polar Lobes

The changes in form of isolated polar lobes have been described in *Dentalium*, by Wilson (1904*b*) and in *Ilyanassa*, by Morgan (1933, 1935). In *Dentalium*, the isolated lobe usually constricts periodically to form lobe-like structures by a process which simulates the formation of polar lobes in the whole egg. There are three such constrictions, and they occur at approximately the same time as do the cleavages of the whole egg. One case is described in which the final constriction gives rise, not to a temporary lobe, but to a distinctly separated portion of cytoplasm. Wilson interprets the first of these changes as the formation of lobes within the isolated polar lobes, at the time when the whole egg normally forms lobes. The final stage he regards as a permanent division of the isolated lobe into two at the time of the fourth cleavage of the whole egg, in which the material of the polar lobe no longer forms a temporary polar lobe, but is permanently cut off by a cell division. Morgan argues against a literal interpretation of the form changes in the isolated lobe as lobes. In *Ilyanassa*, he finds that: (1) The changes in the lobe are not strictly synchronous with the cleavage of the whole egg. (2) The constrictions in isolated lobes come and go at least three times, whereas in the whole egg, the lobe would appear only once more. (3) The change in shape does not, strictly speaking, give a reduced picture of the changes in the whole egg; and, (4) the form changes in the lobe resemble more the process of

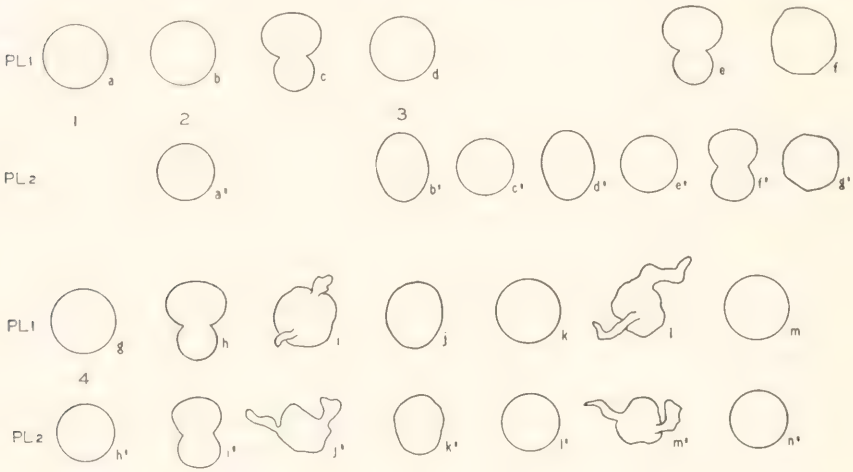


FIG. 5. Form changes in isolated polar lobes. *PL1*, first polar lobe. *PL2*, second polar lobe. Numerals 1–4 indicate the time of the first four cleavages of the whole egg. Explanation in text.

micromere constriction rather than lobe formation. Therefore, Morgan interprets the later changes of the isolated lobe as related to the constriction of micromeres, and not to the formation of polar lobes.

First Polar Lobe.—Isolated first polar lobes of *Sabellaria* were observed continuously for two to three hours. Although, as both Wilson and Morgan found, the behavior is somewhat variable, most lobes show a remarkable constancy in their changes. Of the 31 isolated lobes studied, 25 were of the type drawn in Fig. 5; the other six were more or less variable.

No change in the shape of the isolated lobe occurs at the time of the first cleavage of *E-PL1*, i.e., at the time of the second cleavage of the whole egg (Fig. 5, *b*). About ten to fifteen minutes after the first cleavage of *E-PL1*, the lobe is deeply constricted to form a definite lobe-like structure (Fig. 5, *c*). At the time of the second cleavage, the lobe is spherical (Fig. 5, *d*), but another deep constriction appears about ten to fifteen minutes later (Fig. 5, *e*). As this second constriction disappears, a slight flattening of the lobe occurs (Fig. 5, *f*). This lasts for a short time, and when *E-PL1* cleaves for the third time, the lobe is again spherical (Fig. 5, *g*). A third constriction forms after the third cleavage of *E-PL1* (Fig. 5, *h*), but before it disappears completely, the entire cell becomes irregular in outline, and long irregular pseudopodia are formed (Fig. 5, *i*). These are later withdrawn (Fig. 5, *j*) and the cell again becomes spherical (Fig. 5, *k*). However, it remains in this condition for only a short time, until the irregular pseudopodia are formed again (Fig. 5, *l*). The extension and retraction of the pseudopodia is not synchronous with cleavage, and continues until the lobe cytolyzes. In several cases, the process was still going on twenty-eight hours after the removal of the lobe; in one case, it was observed up to forty-eight hours after the separation of the lobe.

Second Polar Lobe.—The second polar lobes were removed from five isolated *CD* cells, and their behavior followed continuously for two hours. Four of the five lobes produced fairly deep constrictions twice (Fig. 5). The other produced only the first of the two constrictions; at the time when the second constriction would form, the cell elongated somewhat without constricting. The behavior of the four lobes which formed the two constrictions was fairly uniform. When *CD-PL2* divides for the first time, i.e., at the time of the third cleavage of the whole egg, the isolated lobe elongates slightly (Fig. 5, *b′*) and within a minute is rounded out (Fig. 5, *c′*). A similar elongation forms again, in three of the lobes, in about eight to ten minutes after the first (Fig. 5, *d′*). At about seventeen to eighteen minutes after the first cleavage, the lobe develops a constriction which persists for two minutes (Fig. 5, *f′*). As the constriction disappears, the lobe flattens slightly (Fig. 5, *g′*). Within a few minutes, the second cleavage of *CD-PL2* occurs. Following the second cleavage a second constriction forms (Fig. 5, *i′*), and as it disappears the cell becomes irregular and gives rise to pseudopodia (Fig. 5, *j′*). The behavior of the pseudopodia is similar to those of isolated first lobes (Fig. 5, *j′–n′*).

The constrictions formed by the isolated first and second polar lobes resemble in appearance the polar lobes formed by the dividing ovum. Although these constrictions do not occur at the same time as the cleavages of the ovum, they must, in some way, be related to events taking place in the egg during division. This is brought out by a comparison of the time elapsing between successive cleavages of the lobeless egg and the constrictions of the isolated lobe, in the twenty-five cases where the first polar lobe was removed and the five in which the second lobe was removed. The average time between the first and second cleavages of *E-PL1* is 20.6 minutes and that between the first and second constrictions of the isolated first lobe 18.6 minutes. The time between the second and third cleavage of *E-PL1* is 24.6 minutes and that between the second and third constriction is 21.2 minutes. For the isolated second lobes, the average time between the two constrictions is 21.8 minutes, while the interval between the first and second division of *CD-PL2* is 21.0 minutes. The changes in the form of the isolated lobes are apparently synchronous with the cleavages of the ovum, except that all events in the isolated lobes are pushed back by a delay in the appearance of the initial constriction. This delay is approximately ten to fifteen minutes for the isolated first lobe and about seventeen to eighteen minutes for the isolated second lobe.

On the basis that the periodic constrictions formed by isolated polar lobes are correlated with cytoplasmic changes occurring in whole eggs at the time of the cleavages in which polar lobes are formed, we would expect the isolated second lobe to form one fewer constriction than the isolated first lobe. The isolated first lobe forms three constrictions; the isolated second lobe two. And, as would be expected, the time elapsing between the first and second constrictions of the isolated second lobe (21.8 minutes) is almost identical with the time between the second and third constrictions of the isolated first lobe (21.2 minutes). However, certain differences between the behavior of the isolated lobes and that of polar lobes of the whole egg must be noted. The whole egg forms two polar lobes after the formation of the first lobe, and one after the second. The isolated first lobe, however, forms three constrictions and the isolated second lobe two.

Although there is a decrease in size of successive lobes in the whole egg, the constrictions of the isolated lobe are all of approximately the same size. But the facts to be emphasized are: (1) that there reside in the isolated polar lobes materials which take part, independently of the nucleus or the mitotic apparatus, in reactions affecting the tension at the surface of the cell, and (2) that these reactions in isolated lobes occur synchronously (if we discount the initial delay) with events in the whole egg, or in the egg from which the polar lobe has been removed.

TABLE II

Differentiation of larvæ after transplantation of polar lobes and blastomeres.

Type of Operation	Number Operated	Number Survived	Larvæ with:			Larvæ with:			Larvæ with:		
			0 Apical Tuft	1 Apical Tuft	2 Apical Tufts	0 Sets of Bristles	1 Set of Bristles	2 Sets of Bristles	0 Groups of Apical Cilia	1 Group of Apical Cilia	2 Groups of Apical Cilia
A. Polar lobe transplants											
Transplant *Host*											
PL1 Whole egg	26	23	7	16	0	8	14	0	7	15	0
AB	35	32	32	0	0	20	0	0	5	14	0
E-PL1	15	14	14	0	0	10	0	0	3	7	0
PL2 Whole egg	6	5	0	5	0	2	2	0	1	3	0
AB	6	6	6	0	0	3	0	0	0	3	0
B. Transplantation of blastomeres to whole egg											
Transplant											
CD	9	9	0	2	7	0	1	6	1	5	0
C	10	10	0	4	6	0	8	0	2	6	0
D	16	14	2	10	0	0	4	10	2	12	0
AB	11	11	2	9	0	2	8	0	1	4	5
C. Transplantation of blastomeres to *E-PL1*											
Transplant											
CD	16	16	2	11	0	2	12	0	5	9	0
C	20	18	6	12	0	14	0	0	5	8	0
D	15	15	15	0	0	0	13	0	4	9	0
CD-PL2	4	3	1	2	0	2	0	0	0	2	0
AB	12	11	11	0	0	9	0	0	0	3	6
Whole egg	3	3	1	2	0	0	3	0	0	1	2
D. Fusions of blastomeres											
Combination											
CD, CD	20	19	0	11	8	2	2	11	15	0	0
CD, D	9	8	0	7	0	1	1	5	7	0	0
CD, C	6	4	1	2	1	0	2	0	2	0	0
C, C	4	3	0	2	0	2	0	0	2	0	0
AB, AB	12	10	10	0	0	8	0	0	1	2	5
3 *AB*'s	3	2	2	0	0	1	0	0	0	0	1
5 *AB*'s	1	1	1	0	0	1	0	0	Three groups of cilia		

TRANSPLANTATION EXPERIMENTS

Transplantation of Polar Lobes

Transplants of first and second polar lobes were made to whole eggs at the trefoil, two-cell, and four-cell stages, to *AB* blastomeres, and to *E-PL1*. The lobes were placed at the animal pole, at the vegetal pole, and at the equator of the dividing egg. Although fused to the blastomeres, the lobes go through essentially the same form changes as do isolated lobes. When cleavage progresses, the blastomeres may grow over and completely enclose the lobe, or the lobe may remain at one end of the larva. The lobe is still part of the larva when the cilia appear (Fig. 6*A*, *C*). Prototrochal cilia form in all larvæ, and the apical tufts develop only in those which include *C* cells (Table II, *A* and Fig. 6). At about fifteen hours after fertilization, the lobe, more or less completely cytolyzed, is extruded from the larva (Fig. 6, *D*). This is generally followed by a cytolysis of a portion of the embryo, especially in those cases where the lobe was deeply embedded within the embryo. Thus, only 59 out of the 80 larvæ survived beyond twenty-four hours. Where the larva is not greatly damaged by the loss of the lobe, differentiation progresses normally. The *AB* and *E-PL1* larvæ form apical cilia but no post-trochal regions (Fig. 6, *B*). The larvæ produced by entire eggs lose their apical tufts when apical cilia appear, and they develop post-trochal regions with typical bristles (Fig. 6, *F*).

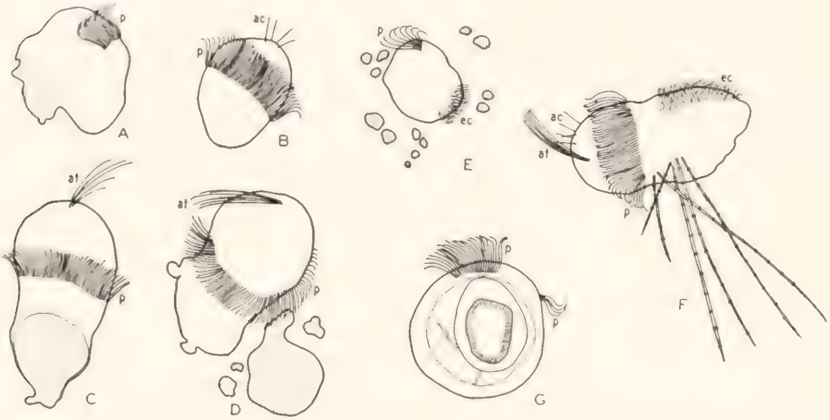


FIG. 6. Differentiation of larvæ after transplantation of polar lobes. *A*. *AB* plus *PL1* larva, 11½ hours. The lobe (stippled) is within the larva. *B*. *AB* plus *PL1* larva, 24 hours. The larva has moved away from the cytolyzed spheres of the ejected lobe. *C*. *E* plus *PL1* larva, 13 hours. The lobe (stippled) is within the larva. *D*. *E* plus *PL1* larva, eighteen hours. The lobe has been ejected. *E*. *AB* plus *PL1* larva, 45 hours. The ejected lobe has broken up into small spheres. *F*. *E* plus *PL1* larva, 26 hours. *G*. *E-PL1* larva, 50 hours. The first polar lobe was in contact with the dividing egg for five hours, at the end of which time it was removed.

In many cases, the polar lobe was stained heavily with Nile Blue sulphate, before being transplanted. The dye diffused from the lobe into the adjacent cells, so that the larval tissues acquired a pronounced blue coloration.

To avoid possible effects of the lobe cytolysis on the differentiation of the larva, the lobe was, in fifteen cases, allowed to remain fused to the dividing cells only until the embryo began to show signs of movement, when it was removed. The contact is long enough so that the blue color of the dye diffuses from the lobe into adjacent cells. The larvæ which develop from such cells are in no essential way altered by contact with the lobe (Fig. 6, *G*).

The presence of the polar lobe, fused to the larva for five hours (when it is removed), or within the larva for eleven hours (at which time it is ejected), does not induce the formation of larval structures.

Transplantation of Blastomeres to Whole Eggs

Table II, *B* summarizes the types of transplants made to whole eggs, at the trefoil, two-cell, and four-cell stages. The transplants are in some cases stained with Nile Blue sulphate before being fused to the host. It is possible to follow the stained region through the first day of development, but beyond this time the dye is not visible. The orientation is varied, but no correlation between any particular orientation and type of development is found.

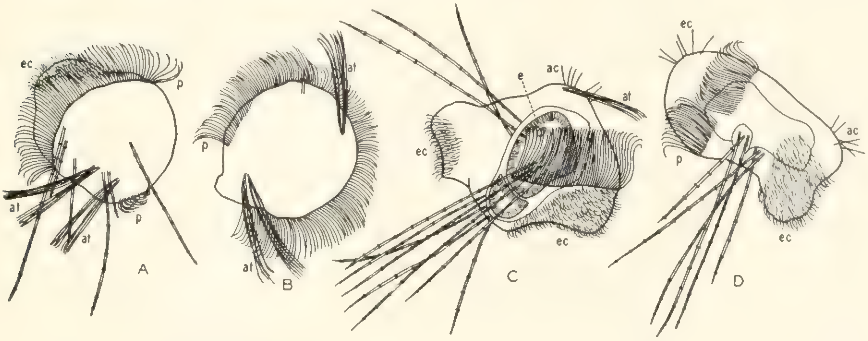


FIG. 7. Differentiation of larvæ after transplantation of blastomeres to whole eggs. *A*. *E* plus *CD* larva, 27 hours. *B*. *E* plus *C* larva, 24 hours. *C*. *E* plus *D* larva, 79 hours. *D*. *E* plus *AB* larva, 40 hours.

The differentiation of the transplanted *AB*, *CD*, *C*, or *D* blastomeres does not bring about the development of additional structures in the host; those structures which are duplicated in the larva arise from the self-differentiation of the transplanted cells. Seven of the nine *E* plus *CD* larvæ show two apical tufts; six (out of the seven surviving) show two sets of post-trochal bristles (Fig. 7, *A*). In six of the ten *E* plus *C* larvæ, two apical tufts are visible (Fig. 7, *B*). Ten of the fourteen *E* plus *D* larvæ possess two sets of bristles (Fig. 7, *C*). The *E* plus *AB* larvæ show no duplication of either the apical tuft or bristles, but five of the ten possess two distinct groups of apical cilia (Fig. 7, *D*).

Transplantation of Blastomeres to E-PL1

The polar lobes are removed from eggs at the trefoil stage, and in their place are put half- or quarter-blastomeres, or whole eggs. In other experiments, transplants are placed at the animal pole, or to the side of *E-PL1*. As in previous operations, the transplanted cells are first stained, either *in toto*, or locally, to mark their polarity. The resultant larvæ are summarized in Table II, *C* and a few of the various kinds are shown in Fig. 8. In all larvæ, the host cells give rise to prototrochal cilia, and in at least 36 of 55, they form apical cilia. They form no apical tuft and no post-trochal bristles. The apical tufts, post-trochal bristles, or supernumerary prototrochal and apical cilia which are present in the larvæ arise only through the self-differentiation of the transplant.

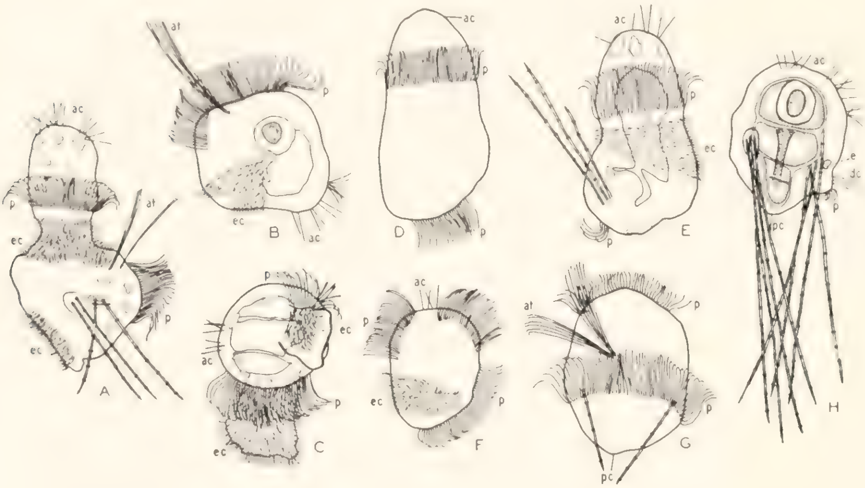


FIG. 8. Differentiation of larvæ after transplantation of blastomeres to *E-PL1*. *A*. (*E-PL1*) plus *CD* larva, 43 hours. *B*. (*E-PL1*) plus *C* larva, 30 hours. *C*. (*E-PL1*) plus *C* larva, 48 hours. *D*. (*E-PL1*) plus *D* larva, 17 hours. *E*. Same larva as shown in *D*, 43 hours. *F*. (*E-PL1*) plus *AB* larva, 54 hours. *G*. (*E-PL1*) plus *E* larva, 25 hours. *H*. Same larva as shown in *G*, 91 hours.

Fusions of CD, C, and D Blastomeres

Thirty-four operations involving combinations of *CD* cells with *CD*, *D*, and *C* blastomeres, and fusions of two *C* cells were performed (Table II, *D* and Fig. 9). Among the 19 larvæ with the *CD*, *CD* combination, 8 show two apical tufts; in 11, only one tuft is distinctly visible. Of the 14 larvæ surviving beyond the first day, 2 fail to develop post-trochal bristles, 2 form one set of bristles, and 11 develop two distinct sets. There are 7 larvæ with the *CD*, *D* combination. Each of these larvæ produces one apical tuft, and 5 of the 7 show two sets of post-trochal bristles. One larva forms one set of bristles and another forms none. Four larvæ of the *CD*, *C* constitution show the following: two apical tufts, 1; one apical tuft, 2; no apical tuft, 1. Two survive long enough to develop bristles; in each there is one set. Only two *C* plus *C* larvæ survive; each possesses only one apical tuft and none forms post-trochal bristles except those which include *D* cells.

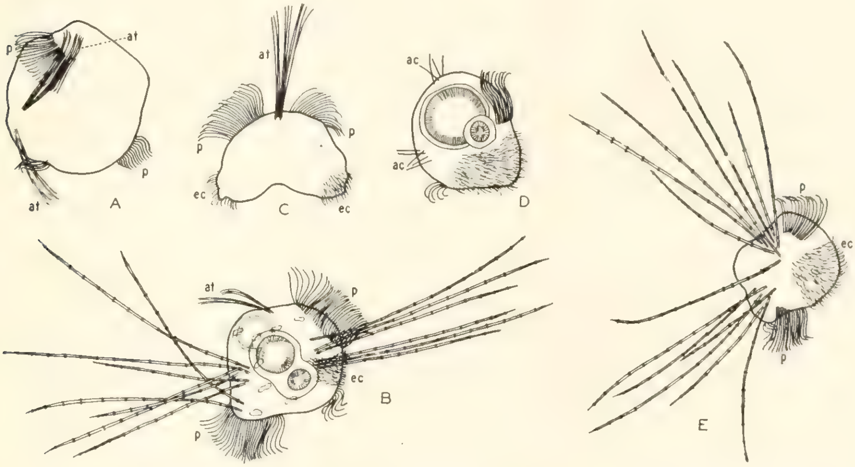


FIG. 9. Differentiation of larvæ arising from fusions of blastomeres. *A*. *CD* plus *CD* larva, 13 hours. *B*. Same larva as shown in *A*, 59 hours. *C*. *C* plus *C* larva, 28 hours. *E*. *CD* plus *D* larva, 55 hours. *D*. *AB* plus *AB* larva, 50 hours.

Fusions of AB Blastomeres

At the time of the first cleavage of isolated *AB* cells, various combinations are effected. Of the larvæ surviving, ten come from fusions of two *AB* cells, two from three *AB*'s, and one from five. Five of seven of the *AB*, *AB* larvæ show two sets of apical cilia, and two show one set; in the single surviving larva from the combination of three *AB*'s, two sets of apical cilia are seen; in the larva from the fusion of the five *AB*'s three distinct sets of apical cilia are visible. Although there is a superabundance of cellular material, none of the larvæ develops an apical tuft and none develops a post-trochal bristle (Table II, *D* and Fig. 9, *D*).

Fusions of Whole Eggs

Two eggs are fused at the trefoil stage or at the two-cell stage. The orientation of the two eggs with respect to each other is varied as follows: (1) the animal-vegetal axes of the ova remain parallel, but the eggs are rotated to different degrees; (2) one of the two eggs is inverted so that the fusion occurs at the animal poles or at the vegetal poles of the two eggs, with the rotation of the eggs varied as in (1).

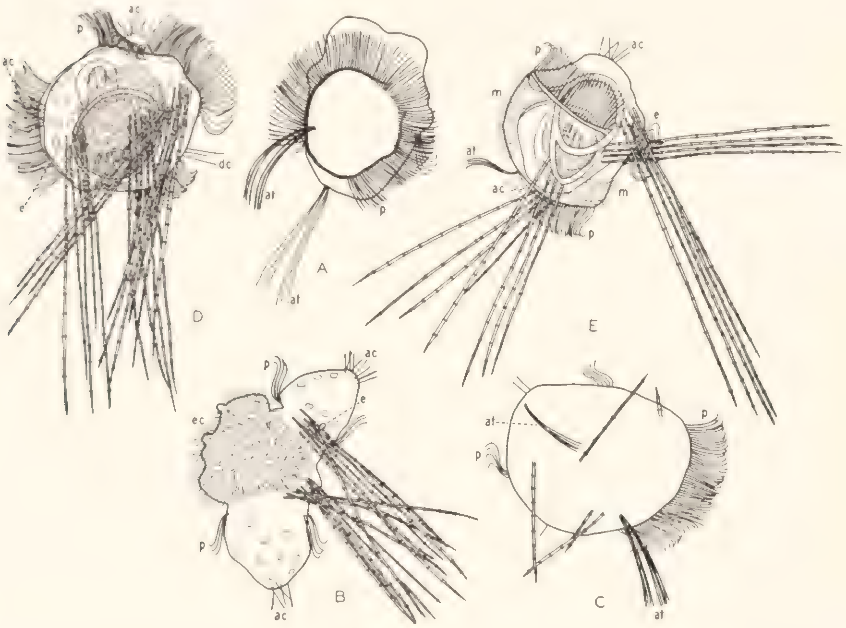


FIG. 10. Differentiation of larvæ arising from fusions of two eggs. *A*. Larva, 23 hours. *B*. Same larva as shown in *A*, 52 hours. *C*. Another larva, 27 hours. *D*. Another larva, 49 hours, with one internal gut. On the outer surface is an area of ciliated cells, devoid of chromatophores. *E*. Another larva, 55 hours, with two internal guts.

Of the twenty-five fusions effected, 23 larvæ are alive at the end of twenty hours of development, and 19 at the end of thirty-five hours. Of the 23, 2 larvæ show no apical tuft, 7 show one tuft, and 11 show two tufts. Among the 19 larvæ, the post-trochal bristles fail to form in one case, 4 larvæ have one set of bristles, and 14 have two sets; the apical cilia are not visible in 2 larvæ, 5 show one group of cilia, and 12 show two distinct groups. In none of the larvæ are there more than two apical tufts, or more than two sets of post-trochal bristles, or more than two groups of apical cilia. Figure 10 includes three advanced larvæ of different types. In one type (Fig. 10, *B*), the endoderm cells of both eggs have been exogastrulated. In another (Fig. 10, *E*), there are two distinct internal guts, of fairly normal structure. In a third type (Fig. 10, *D*), only one internal gut is found. This gut has the typical tripartite character and is no larger than the normal gut. On the outer surface of the larva, a wide, delimited, area is devoid of the chromatophores characteristic of the ectoderm, and the cells of this area are covered with the rapidly-moving cilia, characteristic of the gut cells. The constitution of this area is therefore interpreted as the endoderm cells of one of the eggs. Instead of giving rise to a gut, these cells have become part of the outer covering of the larva; but in spite of their new location, they continue to differentiate as they would normally.

In two instances, three eggs are fused at the two-cell stage. The resultant larvæ develop three sets of post-trochal bristles. In one case, four eggs are fused at the two-cell stage, and the larva develops four sets of bristles.

Discussion of Results

By means of isolation experiments, the independent developmental capacities of early blastomeres of *Sabellaria vulgaris* were determined. These experiments included the usual separation of half- and quarter-blastomeres, and, in addition, the separation of other combinations of cells (*AD*, *BC*, *ABD*, and *ABC*). Also, the effect of the removal of three polar lobes was studied. Table I classifies the 156 surviving larvæ, with respect to the presence or absence of the prototroch, apical tuft, post-trochal bristles, and apical cilia. Prototrochal cilia were present in all larvæ. The apical tuft formed only in those larvæ which included the first polar lobe and the *C* cell. The post-trochal bristles developed only when the three polar lobes and the 1*D* cell were present. The differentiation of apical cilia occurred only when either the *A* or the *B* cell was present. Two of the larvæ were exceptions to this conclusion: one *CD* larva, which developed apical cilia, and one *BC* larva, which developed post-trochal bristles. A third larva, of the *E-PL2* type, formed two apical tufts instead of one.

Having determined the fate of the blastomeres when isolated, the behavior of these same cells when placed in contact with each other atypically, or with isolated polar lobes, was investigated. The twenty-two types of transplantation experiments, including a total of 247 larvæ, are summarized in Table II. Not a single case is found in which the transplant had induced the formation of any particular structure in the host. In any combination of blastomeres, apical tufts develop only when *C* cells are present, post-trochal bristles are dependent upon the presence of *D* cells, and apical cilia form only when either the *A* or *B* cell is included.

In Table III, the larvæ are classified on a different basis. The number of *C*, *D*, and *A* or *B* cells included in the makeup of the individuals are compared with the number of apical tufts, post-trochal structures, and apical cilia. In general, the number of apical tufts is determined by the number of *C* cells present, the number of sets of post-trochal bristles by the number of *D* cells, and the number of groups of apical cilia by the number of *A* (or *B*) cells. The absence of a higher correlation, particularly in the cases of the apical tuft and apical cilia, may possibly be due to several reasons: (1) The larvæ are actively swimming and, especially in small, healthy individuals, such minute structures may be overlooked. (The post-trochal bristles are much more readily seen. The correlation is higher with this structure than

TABLE III

Classification of larvæ developing from transplantation experiments.

Number of *C* Cells	Total No. of Larvæ	Number of Larvæ with		
		0 Apical Tufts	1 Apical Tuft	2 Apical Tufts
0	88	88	0	0
1	95	21	74	0
2	64	3	28	33

Number of *D* Cells *	Total No. of Larvæ	Number of Larvæ with		
		0 Sets of Bristles	1 Set of Bristles	2 Sets of Bristles
0	69	69	0	0
1	63	14	49	0
2	62	4	12	46

Number of *A* (or *B*) Cells	Total No. of Larvæ	Number of Larvæ with		
		0 Apical Cilia	1 Group of Apical Cilia	2 Groups of Apical Cilia
0	26	26	0	0
1	128	35	93	0
2	49	4	15	30

* Two larvæ had 3 *D* cells; both had 3 sets of bristles. One larva had 4 *D* cells; it had 4 sets of bristles.

with either of the other two.) (2) Some of the operated individuals may have been injured and the failure to form a particular structure may be a manifestation of their reduced vitality. (3) Actively swimming larvæ may have some of their cells torn away by the surface at the edge of the drop of water. It is in many cases not possible to determine whether this has occurred. (4) The mechanical effects of the neighboring tissues may prevent the cells from giving rise to the particular structure. If true, this effect might possibly be conceived as a kind of regulatory process.

The most significant feature of the transplantation experiments is that in no instance is the number of apical tufts greater than the number of *C* cells, nor the number of post-trochal regions greater than the number of *D* cells, nor the number of groups of apical cilia greater than the number of *A* (or *B*) cells.

Of special interest are several larvæ which developed from a fusion of two eggs at the two-cell stage. In these larvæ (Fig. 10, *D*), there is present a single gut, of normal size and typical tripartite structure. In one of the two eggs, the cells whose prospective value is gut endoderm have been incorporated into the outer covering of the larva. Although "ectodermal" in the sense of location, these cells continue to differentiate into endoderm, i.e., they do not develop chromatophores and they become ciliated on their outer surface. The self-differentiation of the endoderm in this position indicates, as does the development of exogastrulæ (Novikoff, 1938), the complete independence of endodermal and ectodermal differentiation in *Sabellaria vulgaris*.

Since the polar lobe, as well as any of the quarter- or half-blastomeres, does not affect the differentiation of any cell through contact with that cell, it is not possible to consider the polar lobe an "organizer," in the sense of Spemann (cf. Wilson, 1929, pp. 202–205, and Huxley and deBeer, 1934, pp. 171–172). The experiments of Tyler (1930) have demonstrated that when the first cleavage of the *Chætopterus* egg is made equal—either through the retraction of the polar lobe into the smaller, *AB*, blastomere, or through the cleavage furrow dividing both the egg and the polar lobe equally—then the two half-blastomeres are totipotent. In both types of equal cleaving eggs, the *AB* blastomere receives polar lobe material. If allowed to develop *in toto*, such eggs produce double monsters of the cruciata type. (At the second cleavage, two very small polar lobes may be formed.) When separated, each half-blastomere produces a fairly normal larva. There is in reality no "*AB*" cell; each cell behaves like a *CD*. But there is no evidence that the substance, whose altered distribution changes the prospective value of the cell from that of an *AB* to that of a *CD* cell, is located within the polar lobe. The fact that a double embryo is produced when the polar lobe goes in its entirety into the *AB*, i.e., that the *CD* can produce an embryo without the materials of the polar lobe, indicates the complexity of the situation. That the vegetal hemisphere of the molluscan or annelid egg possesses a particular substance at the time of the first cleavage is well established by the work on *Ilyanassa* (Crampton, 1896), on *Dentalium* (Wilson, 1904*b*), on *Tubifex* (Penners, 1924, 1926), and on *Sabellaria* (Hatt, 1932, and the present paper). In *Tubifex*, if the first cleavage of the whole egg or the *CD* blastomere is made to take place equally instead of unequally (through heat or lack of oxygen)—and the pole-plasms are distributed equally to the two cells—double cruciata monsters are produced (Penners, 1924). A single case was observed in *Sabellaria* in which the first two blastomeres were of equal size. When isolated, each of the blastomeres produced polar lobes at the next two cleavages, and each gave rise to a larva possessing an apical tuft. The fact that each cell forms polar lobes and that the two cells are of equal size indicates that each cell probably received materials from the first polar lobe. It would, then, appear that some substance present in the first polar lobe *does* have the ability to change the course of development of a cell, but that this substance does not act by contact with a cell; it must become a part of the cell. Normally, only the *CD* cell develops an apical tuft since the materials of the lobe flow only into that cell. The exceptional production of apical cilia by a *CD* cell and the appearance of post-trochal bristles in a *BC* combination, mentioned earlier, may be due to a deviation in either of the first two cleavage furrows, with a resultant unusual allocation of materials. The doubling of the apical tuft in the *E-PL2* larva may also be due to an unusual pattern, in which the materials giving rise to apical tufts are separated into different cells.

In the so-called regulative eggs, it is in many cases possible to alter the course of differentiation of a cell by transplanting the cell to a new position in the developing embryo. By varying the stage at which the operation is performed, the time of determination of a structure may be ascertained. Due to the scarcity of similar experiments on mosaic eggs, relatively little information is available concerning the effect of one part of an embryo on another during the course of development, or the existence of inducing, or organizing, regions in these eggs. Penners (1926, 1934) destroyed varying numbers of mesodermal and ectodermal teloblasts of *Tubifex*, at different stages, to test (1) the inter-dependence of ectoderm and mesoderm during development, and

(2) the inducing capacity of the teloblasts. He found that the ectoderm and mesoderm show complete independent differentiation, except for a slight influence of the development of one upon the form and upon the rate of development of the other. The fact that following the destruction of the teloblasts the embryos continue to develop normally indicates that the teloblasts are not organizing centers. Hörstadius (1937*b*) combined various quartets of blastomeres of the sixteen-cell stage in *Cerebratulus;* he found no effect of one layer upon the differentiation of the others. These results are in agreement with those of the present investigation, in which the polar lobe, half-, and quarter- blastomeres are shown to be ineffective in directing the development of *Sabellaria.*

However, in another egg which was thought to be mosaic, the egg of the ascidian, effects of one cell upon the development of the other have been reported. Tung (1934) found that some factor outside the brain is responsible for the formation of the sense organ, in *Ascidiella.* Also, Tung found indications that the adhesive organ is induced. A recent paper by Rose (1937) reports that, similarly, in the egg of *Styela,* the eye spot is induced by the gray macromeres.

SUMMARY

1. Isolation experiments on the egg of *Sabellaria vulgaris* demonstrate that the formation of the apical tuft in partial larvæ is dependent upon the presence of the first polar lobe and the *C* cell; that the post-trochal region develops only when the three polar lobes and the 1*D* cell are present; and that apical cilia form only if the *A* or *B* cell is included.

2. Form changes in isolated first and second polar lobes are described. The early changes are synchronous with the cleavages of the ovum, except that all events in the isolated lobe are delayed.

3. The results of the following transplantation experiments are reported: (*a*) Transplantation of polar lobes. (*b*) Transplantation of blastomeres to the whole egg. (*c*) Transplantation of blastomeres to *E-PL1.* (*d*) Fusions of half- and quarter-blastomeres. (*e*) Fusions of two eggs. In all combinations, complete self-differentiation of individual blastomeres occurs. Apical tufts develop only when *C* cells are present, post-trochal bristles are dependent upon the presence of *D* cells, and apical cilia form only when either the *A* or *B* cell is included.

4. The results of this investigation are compared with those from experiments on other mosaic eggs.

It is with pleasure that the writer expresses his gratitude to Pro-

fessor L. G. Barth for his untiring assistance and constant encouragement throughout the course of this investigation, and to Professor E. B. Wilson for his inspiring interest in the work.

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THE RELATION OF MORTALITY AFTER ENDOMIXIS TO THE PRIOR INTERENDOMICTIC INTERVAL IN PARAMECIUM AURELIA[1]

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Investigators of endomixis in *Paramecium* have frequently observed that animals often die during or soon after endomixis. According to Erdmann and Woodruff (1916), few *Paramecium caudatum* survived endomixis. In *Paramecium aurelia*, Caldwell (1933) found that death occurred 2.85 to 9.40 times more frequently at endomixis than in the middle of the period between endomixes. The present paper is a presentation of the results of a study of one of the factors determining such deaths, namely, the length of the preceding interendomictic interval. As will appear below, when the prior interendomictic interval is unusually long, endomixis results in a greater percentage of deaths than when the interval is of the ordinary duration. Moreover, the greater the interendomictic interval, the greater is the mortality resulting from endomixis, until, after very long intervals, endomixis invariably results in death.

In order to investigate this question, it was essential to have available, simultaneously, lines with normal interendomictic intervals and sister lines with unusually long intervals. This was accomplished by employing recently developed methods of inducing endomixis (Sonneborn, 1937) and of obtaining lines with long interendomictic intervals (Sonneborn, 1938). To induce endomixis, the surplus animals from daily isolation lines of cultivation were collected in a small amount of fresh culture medium and kept at 31° C. for a few days until endomixis occurred. To obtain lines with long interendomictic intervals, daily isolation lines which went into endomixis were replaced by sister lines which had not yet gone into endomixis. In this way, lines with long intervals are selected for study while the lines with shorter intervals are eliminated.

Using these techniques, the following experiment was performed. The vegetative descendants of a single endomictic individual were cultivated as 24 daily isolation lines of descent for 165 days. During this time, all lines that went into endomixis were eliminated and re-

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[1] This work was suggested by Dr. T. M. Sonneborn, to whom I wish to express my sincere appreciation for his helpful advice and assistance.

placed by surplus animals from sister lines; so that at all times this group (I, Fig. 1) consisted of 24 lines that had not been in endomixis since the start of the experiment. At five successive intervals of 21 to 31 days, surplus animals from this group were induced to go into endomixis and the resulting mortality in them was determined. In these groups (I_A, I_B, I_C, I_D, and I_E, Fig. 1), therefore, the mortality after interendomictic intervals of 31, 56, 81, 102, and 125 days, respectively, was ascertained. At the same time that each of these

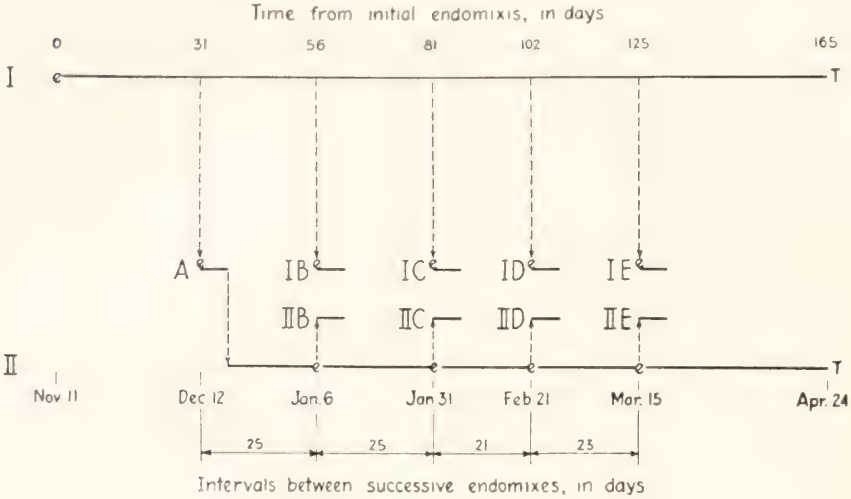

FIG. 1. Plan of Experiment

The horizontal lines (solid) represent groups of isolation culture lines. Long, solid line "I" represents a group of 24 isolation lines from which all endomictics were eliminated. Long, solid line "II" represents a group of 24 isolation lines, interrupted periodically (at points marked *e*) for the induction of endomixis in small mass cultures. The short, solid, horizontal lines represent groups of isolation lines carried for 15 generations. The length of the short lines corresponds to the average number of days that the 15 generations lived in all the groups.

The vertical lines (broken) connect the source groups (represented by long, solid, horizontal lines) with their derived groups (represented by short, solid, horizontal lines). In each case, the derived group began with animals in endomixis taken from mass cultures of animals from the source group.

A, *B*, *C*, *D*, and *E* are the designations given to the derived groups from both I and II. The four derived pairs of groups of isolation lines between which comparisons are made are groups I_B, II_B; I_C, II_C; I_D, II_D; I_E, II_E. Group II was derived from Group I_A by continuing, without endomixis, 24 of the lines of this group.

e stands for climax of endomixis. This symbol is put in on the day on which endomictics were isolated from the mass cultures.

T indicates the time of termination of the experiment.

Time in days, from the initial endomixis in Group I, is shown on the horizontal axis at the top of the diagram. The number of days between successive endomixes is shown at the bottom of the diagram.

groups (except the first) was being studied, there was examined a control group of lines that went through endomixis at the same time, but had instead an interendomictic interval of normal extent (21 to 25 days). The four control groups ($\mathrm{II_B}$, $\mathrm{II_C}$, $\mathrm{II_D}$, and $\mathrm{II_E}$) were obtained by inducing four successive endomixes at the proper time in descendants of the group $\mathrm{I_A}$. Comparisons of mortality were thus made between the following pairs of groups: $\mathrm{I_B}$ (previous interval 56 days) with $\mathrm{II_B}$ (previous interval 25 days); $\mathrm{I_C}$ (previous interval 81 days) with $\mathrm{II_C}$ (interval 25 days); $\mathrm{I_D}$ (interval 102 days) with $\mathrm{II_D}$ (interval 21 days); and $\mathrm{I_E}$ (interval 125 days) with $\mathrm{II_E}$ (interval 23 days).

In this experiment, the animals employed were all descended vegetatively from one which was isolated from a Johns Hopkins stock mass culture of Woodruff's long-lived Yale race. The organisms were cultivated throughout according to the methods described by Sonneborn (1936). Samples of all lines, except those not carried through till the next endomixis, were stained daily to determine the nuclear condition.

TABLE I

Number of fissions from climax of endomixis until death in sixty non-viable lines among one hundred and twenty exendomictic lines followed through until death or the next induction of endomixis.

(In these lines, the maximum interendomictic interval was 71 fissions.)

Number of Fissions until Death	Frequency
1	2
2	3
3	4
4	6
5	13
6	7
7	7
8	6
9	6
10	1
14	1
27	1
29	1
30	1
37	1

Relationship of the Interendomictic Interval to the Percentage of Mortality after Endomixis

The percentage mortality was computed as follows: In each group, each endomictic animal was cultivated as a single daily isolation line until the fifteenth fission after the climax of endomixis. The percentage of lines that died during this period was the percentage mortality for the group. The period of 15 fissions was decided upon as a conservative standard in view of the facts that the process of endomixis itself lasts not more than 9 or 10 fissions and that experience of previous investigators (e.g. Caldwell, 1933) as well as our own showed that deaths rarely occur in the interval from the tenth generation after the climax of one endomixis until the following endomixis. Table I gives the frequency of death at various stages of the interendomictic interval in the material here investigated. As appears from the table, deaths occurred but rarely after the tenth fission.

The results of the experiment are summarized in Tables II to IV. Table II gives a general view of the relation of mortality after endomixis to the length of the previous interendomictic interval. As appears in the table, the percentage mortality increases as the previous interendomictic interval increases. It rose from 32.2 per cent after an interval of 21 days to 100 per cent after an interval of 125 days. The first five intervals, 21, 23, 25, 25, and 31 days are normal interendomictic intervals for Woodruff's stock of *Paramecium aurelia*. The last four intervals of 56, 81, 102, and 125 days are abnormally long intervals for this stock. It will be noted that even in normal interendomictic intervals the percentage of mortality was high.

TABLE II

Relation of mortality after endomixis to length of previous interendomictic interval.

Previous Interendomictic Interval in Days	Percentage of Mortality after Endomixis	Number of Endomictic Lines Observed
21	32.2	90
23	62.5	48
25	59.3	91
25	57.0	79
31	66.6	48
56	71.4	49
81	79.2	53
102	89.3	84
125	100.0	29

It will be observed, from an examination of Table II, that there may be great differences in mortality after endomixis even when the intervals between endomixes are practically the same. Such a difference appears between the percentages of mortality after the 21- and 23-day intervals when the percentages of mortality were 32.2 per cent and 62.5 per cent, respectively. Obviously there are factors other than interendomictic interval involved in the production of mortality. Environmental factors probably play an important rôle here. Therefore, it was essential, for the purposes of this study, to make simultaneous comparisons of groups kept under identical cultural conditions.

The results of such simultaneous comparisons are given in Table III. There the percentages of mortality from endomixis after four abnormally long interendomictic intervals are compared with the percentages of mortality from concurrent endomixes after four normal intervals. Thus, when the interendomictic interval was 56 days, there was 71.4 per cent mortality, as compared with 59.3 per cent in the concurrent group with a normal interval of 25 days. When the interval was increased to 81 days, the mortality was between 79.2 and 84.0 per cent (the exact figure depending upon how many of 20 animals that died without dividing—and hence without a determination of whether they had been in endomixis—were in endomixis), as compared with 57.0 per cent in the concurrent group with a normal interval of 25 days. When the interval was still greater, 102 days, the mortality likewise increased to 89.3 per cent as compared with 32.2 per cent in the controls with a normal interval of 21 days. Finally, when the interval reached 125 days, the mortality was 100 per cent, as compared with 62.5 per cent in the controls with a normal interval of 23 days. The effects of still greater intervals could not be studied because efforts to induce endomixis at intervals of 148, 158, and 161 days all failed; all but a few of the animals in the induction cultures died within 48 hours.

TABLE III

Relation between length of previous interendomictic interval and percentage of mortality after endomixis.

Groups with Normal Interendomictic Intervals			Groups with Long Interendomictic Intervals		
Name of Group	Previous Interendomictic Interval in Days	Percentage Mortality after Endomixis	Percentage Mortality after Endomixis	Previous Interendomictic Interval in Days	Name of Group
II_B	25	59.3	71.4	56	I_B
II_C	25	57.0	79.2	81	I_C
II_D	21	32.2	89.3	102	I_D
II_E	23	62.5	100.0	125	I_E

The results of the experiment, as summarized in Tables II and III, show clearly that as the time between two successive endomixes increases, the percentage of lines that are unable to survive the second endomixis also increases until eventually no animals can survive.

Relationship of the Interendomictic Interval to the Number of Generations between Endomixis and Death

The relationship between the length of the previous interendomictic interval and the number of generations which the non-viable exendomictic lines lived after the climax of endomixis is shown by the data in Table IV. When the previous interval was of normal extent, i.e. about 25 days, as was the case in the control groups, the mean number of generations which the lines lived after the climax of endomixis remained fairly constant, ranging only from 5.0 to 5.9 generations.

TABLE IV

Length of life in number of generations from climax of endomixis until death, in the non-viable exendomictics, in relation to prior interendomictic interval. (The number dying without fission was not determined in experiments 1 and 2.)

Experiment	Group	Interendomictic Interval in Days	Number of Generations from Climax of Endomixis until Death																Total Non-viable Exendomictics	Mean
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1	$\mathrm{II_B}$	25	?	1	2	3	5	15	7	8	8	4	1						54	5.9
	$\mathrm{I_B}$	56	?	1	1	7	9	5	6	3	2			1					35	4.8
2	$\mathrm{II_C}$	25	?	1	1	5	6	13	7	4	2	1	1	2		1	1		45	5.8
	$\mathrm{I_C}$	81	?	7	3	3	6	9	8	4	1	1							42	4.4
3	$\mathrm{II_D}$	21	3	1	2	3	3	3	7	2	1	1	2	1					29	5.0
	$\mathrm{I_D}$	102	6	1	5	13	18	13	8	4	1	4	2						75	4.4
4	$\mathrm{II_E}$	23	4		3	4	3	5	1	2	2	1	1		1		2	1	30	5.5
	$\mathrm{I_E}$	125	21	1	2	2	2				1								29	0.9

Among the experimental groups the mean number of generations decreased from 4.8 generations when the interval was 56 days to 0.9 generation when the interval was 125 days.

In contrast to the steady increase in mortality rate with increasing interendomictic intervals, the mean survival period shows no such steady change. When the interendomictic interval was twice as great as normal the survival period decreased but little. Even when the interendomictic interval was 3 or 4 times as great as normal, the survival period was decreased, on the average, by only about one fission.

Differences of this magnitude are of doubtful significance because the method of determining when the climax of endomixis occurred involved an uncertainty. No direct observation could be made on the endomictic animals removed from the induction cultures to begin the experimental and control groups; but the next day one or more products of their fission were stained and the nuclear condition recorded. On the basis of the number of fragments of the old macronucleus, their size and the intensity of stain, and the size, form, and intensity of stain of the new macronucleus or its anlage, an estimate could be made of how many fissions had occurred since the climax of endomixis; but such an estimate may often be in error by one or two fissions. For this reason, little significance is attached to the slight differences among the preceding groups in the mean number of generations that the non-viable lines survived.

In the group with the longest interendomictic interval, however, the difference is so great as to be unquestionably significant. After an interendomictic interval of 125 days, the non-viable exendomictics went through only one-sixth as many fissions as the non-viable exendomictics with normal prior interendomictic intervals. Indeed, 72.4 per cent of them failed to divide at all after the climax of endomixis. On the third day without fission they were all stained and found to contain fragments of the old macronucleus, but no new anlage or macronucleus. It might be suggested that about three-fourths of the parent lines had lost their micronuclei before endomixis was induced. In such animals only the destructive phases of endomixis can take place as there is no reserve micronucleus from which a new macronucleus can be formed. Loss of the micronucleus after long omission of endomixis has in fact been observed by others in this laboratory (unpublished).

Discussion

1. In view of the complex and superficially paradoxical relations between endomixis and mortality, it has sometimes been held that endomixis is neither a definite phenomenon nor a normal one, but that it is a pathological response of the organism to adverse conditions, the type of response and its consequences varying with the degree of unfavorableness of the environment. In the present work, effects due to differences of environment were avoided by systematically exchanging culture medium between the various groups compared and by restricting comparisons to groups examined at the same time under the same cultural conditions. Thus, the differences in mortality after endomixis were not consequences of environmental differences. Nor were they due to the cumulative action of unfavorable conditions, for in

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each case the groups compared had been subjected equally long to the same conditions. The differences in mortality after endomixis must therefore have been due to the difference in the prior interendomictic interval. The fact that mortality is increased after longer intervals shows that the frequent occurrence of endomixis is an advantage to the stock. In view of this, the interpretation of endomixis as pathological is untenable. Similar conclusions were reached by Kimball (1937) from a study of the precise ratios in which sex segregates after endomixis in this species.

2. The view that Protozoa are potentially immortal and that natural death does not exist among them became popular during the last quarter of the nineteenth century. It has long been known, however, that while a race as a whole may be potentially immortal, certain members of the race are doomed, from internal causes, to die. Thus, Jennings (1913) and others showed that conjugation often resulted in unavoidable death. Erdmann and Woodruff (1916), Jennings, Raffel, Lynch, and Sonneborn (1932), Raffel (1932), Caldwell (1933), Sonneborn and Lynch (1937), and others have shown that endomixis likewise often results in death. Woodruff (1917), Sonneborn (1935), and Jennings and Sonneborn (1936) have shown that long omission of endomixis ultimately results in death. To these intrinsic causes of death in *Paramecium* the present paper adds another: The mortality at endomixis is directly proportional to the preceding interval without endomixis.

SUMMARY

1. Using an interendomictic interval of 20 to 30 days as a standard, it was shown that intervals approximately two, three, four, and five times this long resulted in progressive increases in mortality after endomixis until 100 per cent mortality occurred.

2. At intervals greater than this, the animals died before endomixis could be induced under conditions favorable for its induction.

3. The mean number of generations which non-viable lines survived the climax of endomixis was 5.0 to 5.9 generations when the previous interval was of normal extent. After an interval of 125 days, survival dropped greatly to a mean of 0.9 generation.

4. The results are shown to disagree with current interpretations of mortality at endomixis based on the concept of endomixis as a pathological process.

5. The results show that unusually long interendomictic intervals are, like other previously known conditions, a cause of "natural death" in Protozoa.

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THE EFFECT OF SHORTER THAN NORMAL INTERENDOMICTIC INTERVALS ON MORTALITY AFTER ENDOMIXIS IN PARAMECIUM AURELIA

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In the preceding paper, Pierson (1938) has shown for *Paramecium aurelia* that mortality after endomixis is directly proportional to the length of the interendomictic interval, when the latter is longer than normal. The present paper examines the same question when the interendomictic intervals are shorter than normal.

The same race of *Paramecium aurelia* is examined in both studies. All individuals in the present study were descended without endomixis from one taken from a stock mass culture of this race on February 22, 1936. The methods of culture and of induction of endomixis were similar to those employed by Pierson.

The experiment was performed as indicated in Fig. 1. From a single endomictic individual, 24 daily isolation lines of cultivation were followed for 28 days (Group I, Fig. 1). During this time, samples of each line stained daily showed that no endomixis occurred. On the eighth and sixteenth days after the initial endomixis, the surplus animals from the isolation lines were collected in a mass culture and placed at 31° C. In each case, a high percentage of individuals went into endomixis after 48 hours in these conditions. From each of these, a group of endomictic individuals was isolated and cultivated (Groups II and III, Fig. 1). On the twenty-sixth day after the initial endomixis, three mass cultures were set up, each consisting of the surplus animals from one of the three groups under cultivation. These cultures were placed at 31° C. and all contained numerous endomictic individuals two days later. From each of the three cultures a group of endomictic individuals was isolated and cultivated. These three groups of animals were thus all in endomixis at the same time, but they differed in the interval since the last preceding endomixis: in one, the interval was 10 days; in the second, 18 days; and in the third, 28 days.

The three groups will be designated the 10, 18, and 28-day groups, in reference to their prior interendomictic intervals. The 10-day group was begun with 192 individuals from the culture in which endomixis had been induced; but only 51 of these were in endomixis, as determined by staining products of their fissions on the next day. From each of these 51 individuals, a single daily isolation line was cultivated until it died or until 15 successive fissions had taken place. Of the 51 lines, 48 lived through the 15 fissions and three died after 5 to 12 fissions, giving a mortality rate of 5.9 per cent.

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[1] The author is grateful to Dr. T. M. Sonneborn who suggested the problem and offered his advice throughout the experiment, and to Bernice Pierson and Nathaniel Finkelstein for their kind assistance.

The 18-day group was begun with 144 individuals from the induction culture; but only 94 of these were in endomixis, as subsequently determined. Of these 94 endomictic individuals, 74 lived through the following 15 fissions, and 20 died after 1 to 7 fissions, giving a mortality rate of 21.3 per cent.

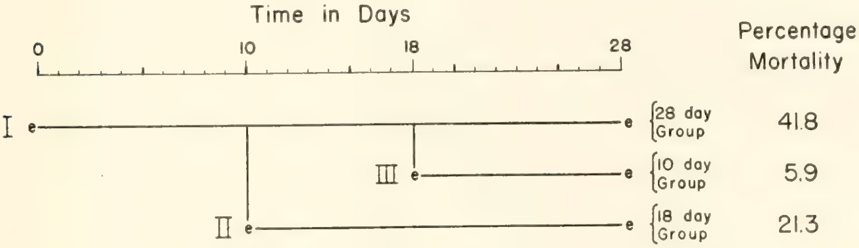

FIG. 1. Plan of Experiment

The three horizontal lines, I, II, and III, represent groups of isolation culture lines carried without endomixis for 28, 18, and 10 days, respectively. The vertical lines connect the source group (horizontal line I) with the two derived groups (horizontal lines II and III). In each case the derived group began with animals in endomixis taken from mass culture of animals from the source group.

e stands for endomixis.

The upper line shows the time in days since the initial endomixis in Group I.

The percentage mortality following endomixis in each group is shown to the right of the line representing that group.

The 28-day group was begun with 144 individuals from the induction culture. Of these, 110 were shown by subsequent staining of their descendants to have been in endomixis. These 110 endomictic individuals were cultivated in the same way as were those of the other two groups. In this group, 64 lived through the 15 fission period of observation and 46 died after 0 to 13 fissions, giving a mortality rate of 41.8 per cent.

Thus, the group with a normal interendomictic interval of 28 days suffered a mortality rate of 41.8 per cent after endomixis, as compared with 21.3 per cent and 5.9 per cent mortality following abnormally short interendomictic intervals of 18 and 10 days, respectively.

The results therefore extend those of Pierson to include abnormally short as well as abnormally long interendomictic intervals. Throughout the entire range of intervals investigated, the mortality after endomixis is directly proportional to the extent of the preceding interendomictic interval.

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DIPLOIDS FROM UNFERTILIZED EGGS IN HABROBRACON

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FEMALES

The occurrence of impaternate females (females from unfertilized eggs) in the parasitic wasp *Habrobracon juglandis* (Ashmead) has previously been reported (Speicher, 1934). Such females occur sporadically from various virgins, and regularly constitute about 1 per cent of the F_2 population from F_1 virgins produced by outcrossing females from tapering or reverted tapering stocks. The hypothesis was offered that these diploid impaternate females might be produced by the failure of the second maturation division in the unfertilized egg and, carrying two chromatids from a single tetrad, would provide material for studying the mechanics of crossing-over.

When F_1 virgins were heterozygous for recessive factors, F_2 impaternate females occurred in the ratio of one homozygous dominant, to two heterozygotes, to one homozygous recessive for each locus involved. This 1 : 2 : 1 ratio was at variance with results obtained in other organisms where more than one strand may be recovered from a single tetrad, notably *Drosophila* (Anderson, 1925) and *Neurospora* (Lindegren, 1933). Here the first maturation division is reductional at the spindle fiber and in dyads from that division the homozygosis of any locus depends upon the amount of crossing-over between it and the fiber, and hence is a function of its location along the chromosome. In *Habrobracon*, except for the locus of the sex-linked factor fused (Whiting and Speicher, 1935), the amount of homozygosis was consistently 50 per cent for all loci tested, even including those of two recessives known to be linked and separated by a distance of ten units.

It was realized from the beginning of the work that other hypotheses could be advanced to explain the formation of impaternate females. And it was apparent that the 1 : 2 : 1 ratio obtained would be expected for all loci if the two homologous chromosome strands of an impaternate female came from two tetrads, independent and com-

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[1] The authors are indebted to Professor P. W. Whiting of the University of Pennsylvania for the use of special microscopic equipment which was supplied in part by a grant to him from the Elizabeth Thompson Science Fund.

pletely reduced, rather than from a single tetrad which underwent only partial reduction. As an investigation of oögenesis in *Habrobracon* had already been begun (Speicher, 1936), it seemed best to suspend further genetic studies until the method of formation of impaternate females could be determined cytologically. Those findings are here reported for the first time.

Since impaternate females had been shown to be genetically diploid, whereas their sibs are haploid, a cytological analysis of their formation seemed practical. According to past work on other forms at least three basic hypotheses could be considered. First, one of the two maturation divisions of an unfertilized egg could be suppressed, as was observed by Silvestri (1908) in the wasp *Prospalta*. This would leave a cleavage nucleus containing the diploid number of chromatids, which would restore the diploid number of chromosomes if the homologues separated. Second, fusion of two reduced egg nuclei present in a binucleate egg also would restore the diploid condition. Third, the egg might originate as a tetraploid, undergo reduction and thus become diploid. A fourth hypothesis, the doubling of chromosomes in the haploid egg during cleavage, is eliminated because tests have shown that impaternate females may be genetically heterozygous.

TABLE I

Progeny from No. 25/reta virgin females.

F_1 virgin ♀ ♀	1	2*	3	4	5	6	7*	8	9	10	11
Sons	83	0	91	26	95	97	5	67	29	113	63
Daughters	1	2	0	0	5	0	0	0	0	0	7
Eggs collected	49	3	50	36	46	61	11	29	48	56	51

* Died before completion of experiment.

The first two theories were tested together. Over 300 eggs were collected from virgin females produced by crossing reverted tapering and stock 25. They were fixed at first cleavage prophase and stained by the Feulgen whole-mount method. The same females produced collectively 15 impaternate daughters among 724 sons, over 2 per cent of the total. Among the eggs studied approximately the same proportion would be expected to show cytological differences if either theory were correct. Suppression of a maturation division would result in a decrease in the normal number of polar nuclei formed at the egg margin. A binucleate egg would be expected to show two groups of polar nuclei, totaling twice the normal number. An examination of all eggs revealed none showing either of these two differences.

Treatment of the third hypothesis requires chromosome counts during maturation; accordingly it was necessary to obtain a new lot of eggs fixed at an earlier stage than the above. It had been noticed previously that some virgin females produced impaternate daughters in small groups while others in the same experiment produced no daughters whatever. Inclusion in the data of the offspring from the latter virgins lowers the percentage of impaternate females among total offspring. It was therefore possible to raise the expected percentage of exceptional eggs by selecting eggs only from virgins known to be producing impaternate daughters. This was accomplished as follows. Eleven females from a cross of reverted tapering by stock 25 were placed with host caterpillars. Eggs laid upon the caterpillars during seven consecutive days were fixed at first anaphase and temporarily stored in alcohol. Eggs laid at night over the same period were allowed to develop, in order to indicate which of the eleven females were thelytokous. Results are shown in Table I.

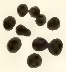

Fig. 1.

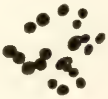

Fig. 2.

FIG. 1. First anaphase of normal egg. × 3,000.
FIG. 2. First anaphase of tetraploid egg. × 3,000.

Seven females, producing a total of 428 sons, had no daughters, while the remaining four produced 241 sons and 15 daughters. The 149 eggs collected from the latter four females were then stained by the Feulgen whole-mount technique. One hundred and ten of these were in condition to study; the remainder were either lost in handling or were collapsed. Ninety-eight eggs were unquestionably diploid, seven were unquestionably tetraploid and five more were questionably tetraploid. The clear cases of tetraploidy showed twenty chromosomes, presumably bivalents although individual chromatids have never been observed in *Habrobracon* oögenesis due probably to their small size, moving to one or to each pole. Normal diploid eggs show only ten chromosomes going to each pole, Figs. 1 and 2.

These cytological data, and the fact that impaternate females come in groups from certain mothers suggest the probability that production of tetraploid eggs, as developed from patches of tetraploid ovarian tissue, is responsible for the appearance of diploid impaternate females among haploid brothers.

MALES

Diploid males of biparental origin have been reported repeatedly in *Habrobracon*. Since diploid females are produced by virgins as a result of tetraploidy in egg cells, it seems plausible that diploid impaternate males may be produced in the same way. According to Whiting's scheme of sex-determination in *Habrobracon* (Whiting, 1933) those eggs which were diploid after reduction and contained sex chromosomes X and Y would produce impaternate females, while those which contained chromosomes XX or YY would produce diploid impaternate males. If distribution of chromatids is random the number of diploid females and males produced should be equal.

TABLE II

Data from tests for diploid impaternate males.

F_1 ♀♀	F_2 haploid ♂♂, A or a	F_2 impaternate ♀♀		
		AA	Aa	aa
o^s/o^i	6222	6	10	5
Le/le	6070	17		6
St/st	4408	4	2	0

Three experiments were set up in an effort to produce diploid impaternate males that could be distinguished genetically from their haploid brothers. Since the highest percentages of impaternate F_2 diploids had previously resulted from outcrosses of tapering and reverted tapering females, tapering was again used as the maternal stock in one experiment. In the other two it was necessary to introduce recessive factors into the cross through the females. In order to insure the occurrence of thelytoky in these cases the recessives were repeatedly bred up to reverted tapering, and a stock related to it but having the desired genetic constitution was then derived and supplied the females for the parental crosses. In each experiment F_1 virgins were produced carrying both of two allels that give rise to a distinct phenotype when they are heterozygous.

Results are given in Table II where the first column shows the pairs of allels used to make the heterozygotes recognizable phenotypically, the second column gives the number of normal haploid males in the F_2, and the third column gives the classes of impaternate females. Theoretically diploid impaternate males should have occurred in the same numbers and in the same genetic ratio (1 homozygous dominant, AA : 2 heterozygotes, Aa : 1 homozygous recessive, aa) as did the impaternate females, although among the males only the heterozygotes (Aa) would have been distinguishable from their normal haploid brothers.

No diploid males were found and the experiments are published at this time because the stocks with which they might be repeated are no longer extant and the work is finished unless or until the thelytokous tendency reappears in *Habrobracon*. However, results are not considered entirely convincing, nor is the evidence against the occurrence of diploid impaternate males considered conclusive. The following paragraphs tell for each experiment why the tests were not as satisfactory as had been expected.

o^i/o^d.—Wasps heterozygous for the allelomorphic eye colors ivory (o^i) and dahlia (o^d) had previously been distinguished phenotypically by a lighter color than the dark red of homozygous dahlia (Torvik, 1931). In this experiment light dahlia was recognizable in the F_2 females, where it was checked by breeding tests. In the F_2 males, however, the color varied widely, grading from dark to light. Forty males were recorded as having eyes as light as their ten heterozygous sisters.

F_1 virgins in the eye color experiment were also heterozygous for the sex-linked factor fused (*fu*, antennæ and tarsi). Diploid impaternate daughters would be heterozygous for the sex region (XY), and also for fused except where cross-overs had occurred betewen fused and the sex region (10 per cent of the cases). Of the 21 impaternate females produced none was homozygous fused, and wherever it was possible to make breeding tests fused males occurred among their progeny, so that actually no cross-over cases were found. Diploid impaternate males, on the other hand, would be either XX or YY, and homozygous for *fu* or *Fu* except where cross-overs had occurred. Thus almost half the diploid males would carry two fused genes. C. H. Bostian had previously noted that the presence of two fused genes in diploid biparental males frequently made the antennæ shorter than those of fused haploid males where only one fused gene occurs. It was hoped that here extreme shortness of antennæ might serve as an additional tag for diploidy. However, antennal length proved to be too variable to be of any service, grading gradually from long to short.

Le/le.—In heterozygotes for the gene lemon (*le*, body color) pigmentation is normal except for the basal segments of the antennæ which are yellow instead of dark brown. The dominant factor Minnesota yellow (*My*, base of antennae) produces exactly the same phenotype at high temperatures. It is homozygous in reverted tapering stock, and an effort was made to breed it out before this experiment was begun, but its presence was simply hidden by temperature changes. In the F_2 both *My* and *le* segregated. Of the impaternate females with normal body color it was impossible to tell which carried lemon. All but one were recorded as having yellow antennal bases due to the presence of one or the other or both of the mutant genes. If diploid males heterozygous for lemon were present, it was impossible to distinguish them from their haploid Minnesota yellow brothers.

St/st.—The experiment using the stumpy gene (*st*, legs) was abandoned because the number of F_2 impaternate females was so small that the expectancy for heterozygous diploid males fell so low as to make the search unprofitable, in spite of the fact that this was the one case where parental females had been of pure tapering stock.

SUMMARY

1. Cytological evidence shows that unfertilized eggs which give rise to females are tetraploid before reduction and therefore diploid after reduction.

2. No diploid males from unfertilized eggs were found among 16,700 males examined.

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INACTIVATION OF SPERM BY X-RADIATION IN HABROBRACON

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Sterility of the male may be due to lack of sperm, to defective or inactivated sperm unable to fertilize the eggs or to sperm carrying dominant lethals which kill the fertilized eggs.

The wasp *Habrobracon* is convenient material for the study of these types of male sterility. Normally males develop from unfertilized eggs by haploid parthenogenesis, females from fertilized eggs. Absence or inactivation of sperm would result in the production of no daughters and of sons equal in number to those of unmated females. If all the sperm contain dominant lethals no daughters are produced and the fecundity of the mated females is correspondingly reduced. Consequently analysis of differences in biparental ratio and in fecundity indicates the type of male sterility which has been operative.

P. W. Whiting (1937) demonstrated that treatment of sperm with X-ray dosages of 20,000, 40,000 and 75,000 r units produces at least one dominant lethal in every sperm cell. No daughters occur in the progeny and the average number of males produced per day does not equal that to be expected from virgin females, indicating that many eggs are fertilized and die. Sperm treated with 75,000 r units fertilized almost as many eggs as untreated sperm; therefore the treatment apparently did not cause inactivation. However, a slight increase in the average males per day from mates of males treated with 75,000 r units as compared with those from mates of untreated controls suggested the possibility that spermatogenesis might to some extent be stopped and sperm supply decreased.

An experiment carried out with the X-ray equipment at the Marine Biological Laboratory, Woods Hole, Mass., July–August, 1937, was designed to test this hypothesis (Table I). One group of wild-type males (stock 32) was treated with 41,000 r units, another group with 142,000 to 143,000 r units. A third group was untreated. Each male was mated on each successive day to a different female which was

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[1] The author is indebted to the University of Pennsylvania for furnishing laboratory space at the Marine Biological Laboratory during the summer of 1937, and to the Committee on Effects of Radiation on Living Organisms (National Research Council) for technical assistance furnished from a grant to Dr. P. W. Whiting.

segregated for breeding. Unmated females of the same stock were segregated each day as controls. The total number of days of the egg-laying periods of all females of any one group mated on the same day is the basis of the averages of offspring per day.

It was expected that successive matings would exhaust the sperm of the treated males provided that no spermatogenesis took place after the treatment. Such exhaustion does not occur since the last matings of these males produce average males per day equivalent to those from mates of untreated males and lower than the average for the offspring of unmated females.

Table I

Offspring from orange-eyed females (stock 11–o) mated with untreated or with x-rayed wild-type males (stock 32) or bred unmated.

Day of mating	Offspring from untreated males					Offspring from 41,000 r males			Offspring from 142,000 and 143,000 r males		
	Days	+ ♀♀	Per day	o ♂♂	Per day	Days	o ♂♂	Per day	Days	o ♂♂	Per day
1st	152	524	3.44	247	1.62	139	184	1.32	219	464	2.11
2nd	136	671	4.93	203	1.49	129	257	1.99	208	368	1.76
3rd	145	337	2.32	116	.80	120	245	2.04	120	285	2.37
4th	168	487	2.89	174	1.03	137	223	1.62	89	176	1.97
5th	113	303	2.68	103	.91	120	128	1.06	41	71	1.73
6th	93	185	1.99	62	.67	128	112	.88			
7th	117	302	2.58	59	.50	60	44	.73			
8th	100	258	2.58	87	.87	128	194	1.51			
9th and 10th	59	188	3.18	82	1.38	116	128	1.10			
Totals from mated females	1083	3255	3.01	1133	1.05	1077	1515	1.41	677	1364	2.01

Total from unmated females. . . . Days, 344. Males, 1073. Males per day, 3.12.

In the total averages of males per day for all the fraternities from treated, evidence is found of failure of sperm to fertilize and thus kill some of the eggs. The total average of males per day increases with increasing dosage and for 142,000 to 143,000 r units is intermediate (2.01) between the mated controls (1.05) and the unmated controls (3.12).

Inactivation of sperm following higher dosages rather than partial exhaustion of sperm supply due to partial inhibition of spermatogenesis is indicated by the fact that there is no increase in males per day from the later matings. It is probable that even much weaker dosages than those here used completely stop spermatogenesis. Thus we have partial male sterility due to dominant lethals at relatively weak dosages, complete male sterility due to dominant lethals at stronger dosages, and partial sperm inactivation at very high dosages.

The average offspring per day (males) from unmated females (3.12) is less than the average offspring per day (females and males) from females mated to untreated males (4.05). This is attributed to recessive lethals segregating in certain fraternities summarized together in Table I. In other experiments viability of males and females has been about equal.

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THE SEX RATIO IN MELITTOBIA CHALYBII ASHMEAD, GAMETOGENESIS AND CLEAVAGE IN FEMALES AND IN HAPLOID MALES (HYMENOPTERA: CHALCIDOIDEA)

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INTRODUCTION

In the chalcid-fly, *Melittobia*, females develop only from fertilized eggs, males from unfertilized eggs. The total number of males produced by any individual female is approximately only 3 per cent of the total number of offspring—an unusual condition in view of the fact that these males are indispensable for the continued existence of the species. Not only is the proportion of males very low, but even in virgin females, the number of eggs which develop parthenogenetically into males is not, it is estimated, much over 3 per cent of the total number of eggs laid. The remainder of the eggs die and shrivel up.

When an examination revealed that the males in this species are haploid and that we were not dealing with the common type of facultative parthenogenesis which involves a restoration of diploidy in a limited number of azygous embryos, the question that naturally presented itself was: Why does only such a small percentage of eggs possess parthenogenetic potentialities? As a step in the investigation of this problem cytological observations on gametogenesis, fertilization and cleavage are here recorded.

LIFE HISTORY

Melittobias have been much studied both in America and in Europe. Interest in them has centered about their life-history, their polyphagous habit and the striking structural antigeny they exhibit. More recently (Schmieder, 1933), polymorphism and the factors responsible for this phenomenon have been described in females of *M. chalybii.*

These parasites are most frequently found within the cocoons of certain wasps, especially *Sceliphron*, *Chalybion* and *Trypoxylon*. In addition to *M. chalybii*, taken in southern New Jersey, two other species, as yet unidentified, have been studied, one from New Jersey, the other from numerous localities in New Jersey, Pennsylvania, Ohio and Indiana. None of these three species when crossed with one another produced any female offspring but only matroclinous males. The observations in this paper are all based on *M. chalybii* material. The other species were, however, also studied and were found to be, cytologically, like *chalybii*.

In the rearing of these insects, one mated female is placed on a host which has been removed from its cocoon. Feeding and oviposition soon commence and the first 12 to 30 female offspring that emerge oviposit on the same host. Finally, the remainder of the first and the entire second generation (altogether some 500 to 800 offspring) emerge. From 2–5 per cent, usually about 3 per cent, of these are males. Although the proportion of males is small and although their number is still further reduced by the mortal combats which occur among them, practically all the females are eventually fertilized. Indeed, the sex ratio here seems to be an adaptive feature which conserves the food supply of the species for the almost exclusive use of the female sex, the sex which alone serves the dispersal of the species. This method of rearing probably approximates what occurs in nature, except that in nature occasional cross-breeding between different lines no doubt may occur while in the laboratory inbreeding is the rule. The stock now on hand is the seventh successive inbred culture. The number of the generation is not known because of the intermingling of two successive generations in each culture. Of one of the other species, with a shorter life history, the twenty-sixth inbred generation is at hand.

Unmated females, segregated during the pupal stage, either may not oviposit at all or may lay only a very few eggs, perhaps from 1 to 5. Of these eggs one or two may develop into males, or all the eggs may die. In an exceptional instance 30 males were obtained from 2 virgins, but the record states that many dried up eggs were also found in this culture.

While virgin females are disinclined to oviposit, such females, after being mated with a male of a different species, generally oviposit as freely as do normally mated individuals. Apparently the act of mating or the presence of sperm in the seminal receptacle acts as a stimulus to oviposition. In no instance, however, did any females develop from such eggs, but only matroclinous males. In mating males of another species to *chalybii* it is necessary to employ the short-winged polymorphic form of *chalybii* whose copulatory reaction time corresponds more closely to that of the foreign males. This short-winged form, however, deposits eggs in very rapid succession and, even when normally mated, an unusually large proportion of these eggs fails to develop.

In the other two species studied no such difficulty exists and in them it is evident, as in *chalybii*, that only a small proportion, possibly again not more than 3 per cent, develops parthenogenetically into males while all the other eggs die. In one instance 3 females produced over 200 eggs by the sixth day. They were allowed to continue to oviposit until the tenth day but this culture eventually yielded a total of only 20 males. These same females transferred successively to two other hosts, upon which oviposition continued at almost the initial rate, produced on the second host 16 males and on the third host 16 males. The same procedure when carried out with two other females yielded 12 males from the first, 6 from the second and 3 from the third host. No female offspring appeared in any of these cultures.

It is therefore evident that both virgins and females mated to males of another species may produce about the same number of male offspring as do normally mated individuals. Those of their eggs which fail to develop, one is tempted to assume, are those which would have yielded female offspring, had their mothers been mated in the normal way. Without a more adequate statistical analysis, it is not possible to make more definite statements at the present time, especially in view of the high egg mortality often observed even in normal cultures.

Materials and Methods

The *chalybii* material examined consists of: 50 pairs of testes of larvæ and pupæ, which were dissected out on glass slips and fixed as smear preparations with Carnoy-Lebrun's and Bouin's fluids; 25 pairs of testes, dissected out in Belar's solution, fixed mainly in Benda's, Bouin's and Petrunkevitch's fluids and sectioned at 4 to 6 μ in paraffin; 28 pairs of ovaries of pupæ and imagines, dissected out and fixed directly on glass slips with Carnoy-Lebrun's or Bouin's solution; 220 eggs of normally mated females and 154 eggs of females mated with males of another species, all fixed in Carnoy-Lebrun's medium.

The sectioned material was stained with Heidenhain's hæmatoxylin. The smears of testes and of ovaries were stained with Mayer's hæmalum, containing 2 per cent of acetic acid, and by the Feulgen method. For the staining of entire eggs the Feulgen method only was entirely satisfactory. All material was mounted in gum damar.

Observations on Males

Somatic Tissues

In the cells of the nervous system and of the imaginal discs of pupæ and prepupæ of males there were invariably 5 chromosomes, the haploid number (Figs. 1 and 2). This same number was again found in the unfertilized eggs in all stages from early cleavage until hatching (Fig. 13).

Testis

Development of spermatogonial cysts and multiplication of spermatogonia occur during larval life and only during that period are gonial division stages in evidence. In very young pupæ, in which not even the eye spots as yet show any traces of pigmentation, about one-third of the testis is already occupied by spermatids and mature sperm. Cysts containing the youngest spermatocytes (Fig. 3), arranged in the form of rosettes, occupy the anterior end of the testis.

The middle region is taken up largely by spermatocytes in the growth stage and by division figures. During the growth period the nucleus contains a prominent karyosphere and a lightly staining network (Fig. 4). This karyosphere gives way to the fine spireme of the first spermatocyte prophase. As the spireme finally becomes a very open one, the five chromosomes present can easily be distinguished in every cell (Fig. 5). In metaphase plates of spermatocyte divisions (Figs. 6 and 7), it is noted that all five chromosomes are atelomitic. The first division, most clearly recognizable in anaphase (Fig. 8), is abortive and yields only one second spermatocyte. In Fig. 8 and in the figures of the second spermatocyte division (Figs. 9 and 10), only the spindles are shown, which are always clearly distinct from the poorly fixing zone of cytoplasm which surrounds them. The two spermatids formed by division of each second spermatocyte both transform into sperm cells.

Observations on Females

Somatic Tissues

The cells of the nervous system and of the imaginal discs of female prepupæ are always diploid, with 10 chromosomes. The same is true of the follicular cells of the ovary and of the cells of embryos developing from fertilized eggs (Figs. 21 and 34).

Ovary

At the cephalic end of each of the 6 to 8 ovarioles comprising each ovary there is a region in which the oögonia and the future follicle and nurse cells are indistinguishable (Fig. 16). Immediately caudad of this limited region the young oöcytes become recognizable by the synaptic stages which are observable in them at a time when these cells are hardly larger than the neighboring presumptive nurse and follicle cells (Fig. 16). In the post-synaptic spireme (Fig. 22), 5 pachytene threads are always clearly discernible. Following synapsis, the oöcyte undergoes a steady, continuous growth until maturity, while the follicle cells and the nurse cells assume the arrangement characteristic of meroistic ovarioles (Figs. 17–20). During this period of growth the chromosomes become more and more deconcentrated and diffuse (Figs. 23–25). In the course of this change they become at first less sharply outlined and open up, the opening up process beginning near their ends, in a manner suggesting a longitudinal splitting. Finally large open figures are formed which are most frequently in the form of loose rings. Although this ring-like organization is not always determinable for each of the chromatic elements due to their large size, haziness and frequent discontinuity, yet it is evident that the chromosomes never entirely disappear during the growth period of the egg.

Toward the end of the growth period the diffuse chromosomes of the germinal vesicle condense and contract into small chromatic bodies which are perhaps suggestive of the precociously formed abortive maturation spindle often described in the literature. (This stage will require additional study.) Finally, when the egg is ready to leave the oviduct, the nucleus is represented by a single small karyosphere

Explanation of Figures

Figures 3, 4, 8, 9 and 10 are from material fixed with Benda's fluid and stained with Heidenhain's hæmatoxylin.

Figure 6 is from material fixed with Bouin's fluid (B 15) and stained with Heidenhain's hæmatoxylin.

All other figures, on both plates, are from material fixed with Carnoy-Lebrun. Figures 16, 17, 21–24 are from slides stained with acid hæmalum; the remainder from slides stained by the Feulgen method.

Magnification: All figures of individual cells or nuclei (Figs. 1–11, 13, 14, 21–26, 28, 30, 32, 34), × 5,000.

Figures of eggs (Figs. 12, 15, 27, 29, 31, 33, 35), × 500.

Figures of the ovariole (Figs. 16–20), × 400.

Plate I

Fig. 1. Somatic cell of larva, male; late prophase.

Fig. 2. Somatic cell of larva, male; metaphase.

Fig. 3. Young spermatocyte, from a rosette.

Fig. 4. Spermatocyte in the growth stage.

Fig. 5. Spermatocyte in late prophase.

Figs. 6 and 7. Spermatocytes in metaphase.

Fig. 8. First spermatocyte anaphase.

Fig. 9. Second spermatocyte metaphase, lateral view.

Fig. 10. Second spermatocyte anaphase.

Fig. 11. Second oöcyte division, metaphase; from Fig. 12.

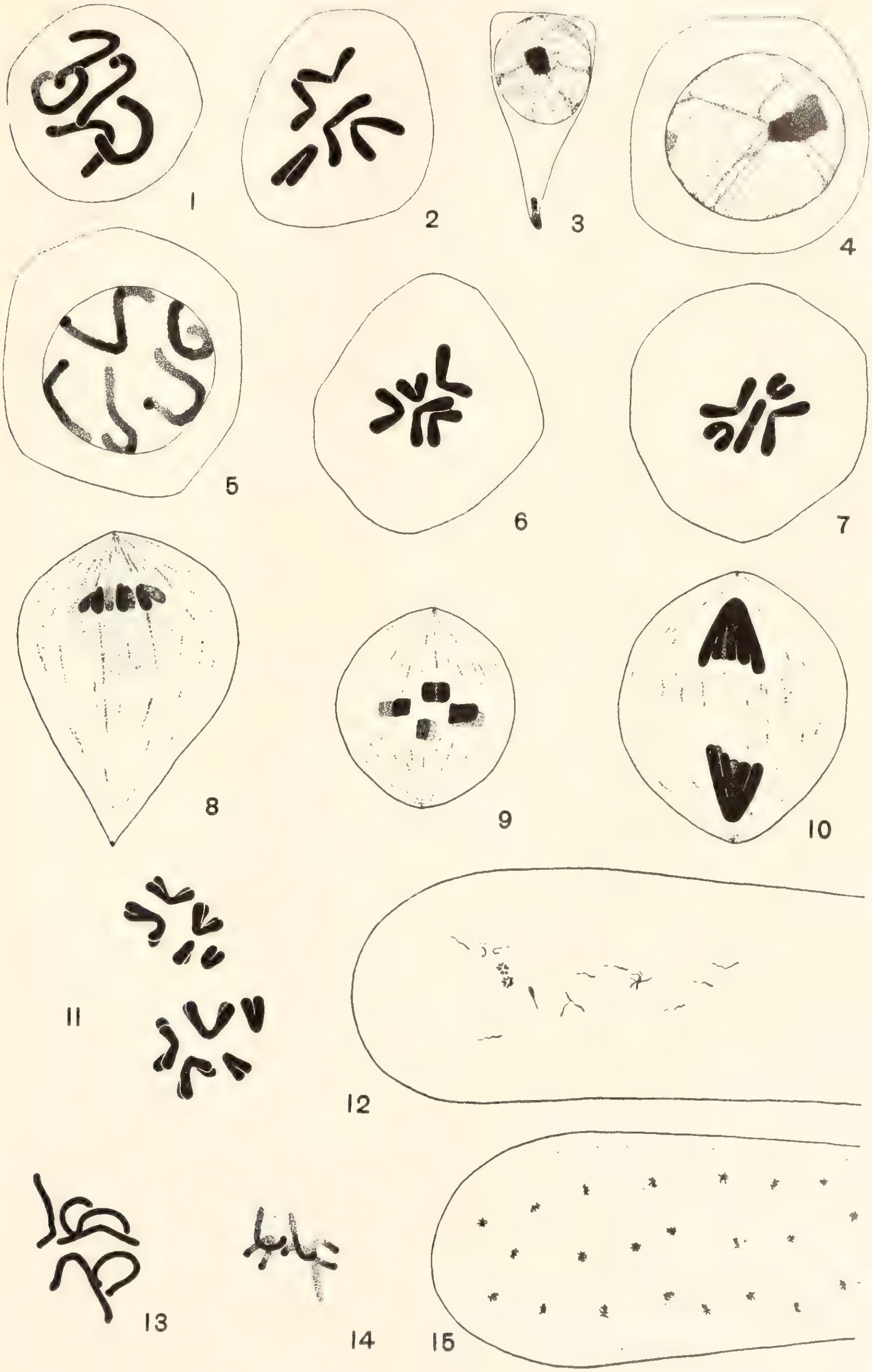
Fig. 12. Egg in second meiotic division, from a female mated with a male of another species. One sperm is within the egg, at the center, and is swollen; 14 additional sperm are adhering to the outer surface of the egg.

Fig. 13. Haploid metaphase of a cleavage nucleus, from Fig. 15.

Fig. 14. Haploid metaphase of a cleavage nucleus, lateral view, from Fig. 15.

Fig. 15. Anterior portion of an egg in the eighth cleavage division; haploid. Only those cells which occupy that half of the egg nearest the observer are represented in the figure.

PLATE I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separated from the deutoplasm of the egg by a thin zone of clear protoplasm (Fig. 20).

In Figs. 16 to 20 representative portions of a complete ovariole are shown, including those from which the nuclei, drawn on a larger scale in Figs. 21 to 25, were selected.

CLEAVAGE IN UNFERTILIZED EGGS

Unmated females deposit only a very few eggs and of these few only a small percentage undergoes development. It is therefore more practicable to study haploid development in eggs obtained from females mated with another species of *Melittobia*, since after such mating oviposition occurs often at a rate comparable to that occurring in normally mated females. The foreign sperm is generally observed as having entered the egg but actual syngamy seems never to occur, or, at least, never to yield viable zygotes. The small percentage of eggs that eventually hatches always produces haploid males of the maternal type.

Eggs fixed ten minutes after oviposition are commonly in the second oöcyte stage, as shown in Fig. 12. Here is seen the foreign sperm which is within the egg and is considerably swollen, as well as an unusual number of additional sperm adhering to the outside surface of the egg.

PLATE II

FIG. 16. Cephalic end of an ovariole, with undifferentiated cells, oöcytes in synizesis, and post-synaptic spireme; nurse cells and follicle cells.

FIG. 17. Portion of an ovariole with young, growing oöcyte and nutritive chamber.

FIG. 18. The same, somewhat older.

FIG. 19. Anterior end of a half grown egg, with the follicle cells.

FIG. 20. Anterior end of a fully grown egg within the ovariole.

FIG. 21. Undifferentiated diploid cell from the ovariole.

FIG. 22. Oöcyte nucleus in post-synaptic spireme stage.

FIG. 23. Oöcyte nucleus, later stage in early growth period.

FIGS. 24 and 25. Oöcyte nuclei during middle growth period; the chromosomes hazy, diffuse, usually more or less ring-shaped.

FIG. 26. Oöcyte nucleus preparing for the first meiotic division, from Fig. 27.

FIG. 27. Egg, less than 4 minutes after oviposition; the sperm is slightly enlarged and near the center, the oöcyte nucleus near the periphery.

FIG. 28. Egg chromosomes at the end of the first oöcyte division, from Fig. 29.

FIG. 29. Egg, less than 15 minutes old, with first meiotic division completed. The sperm is now swollen and at the center of the anterior part of the egg; the egg chromosomes are near the periphery, those of the polocyte nearest the egg surface.

FIG. 30. First cleavage prophase of a fertilized egg, from Fig. 31; two chromosome groups of five each are distinguishable.

FIG. 31. Egg in prophase of first cleavage; the polocytes are at surface.

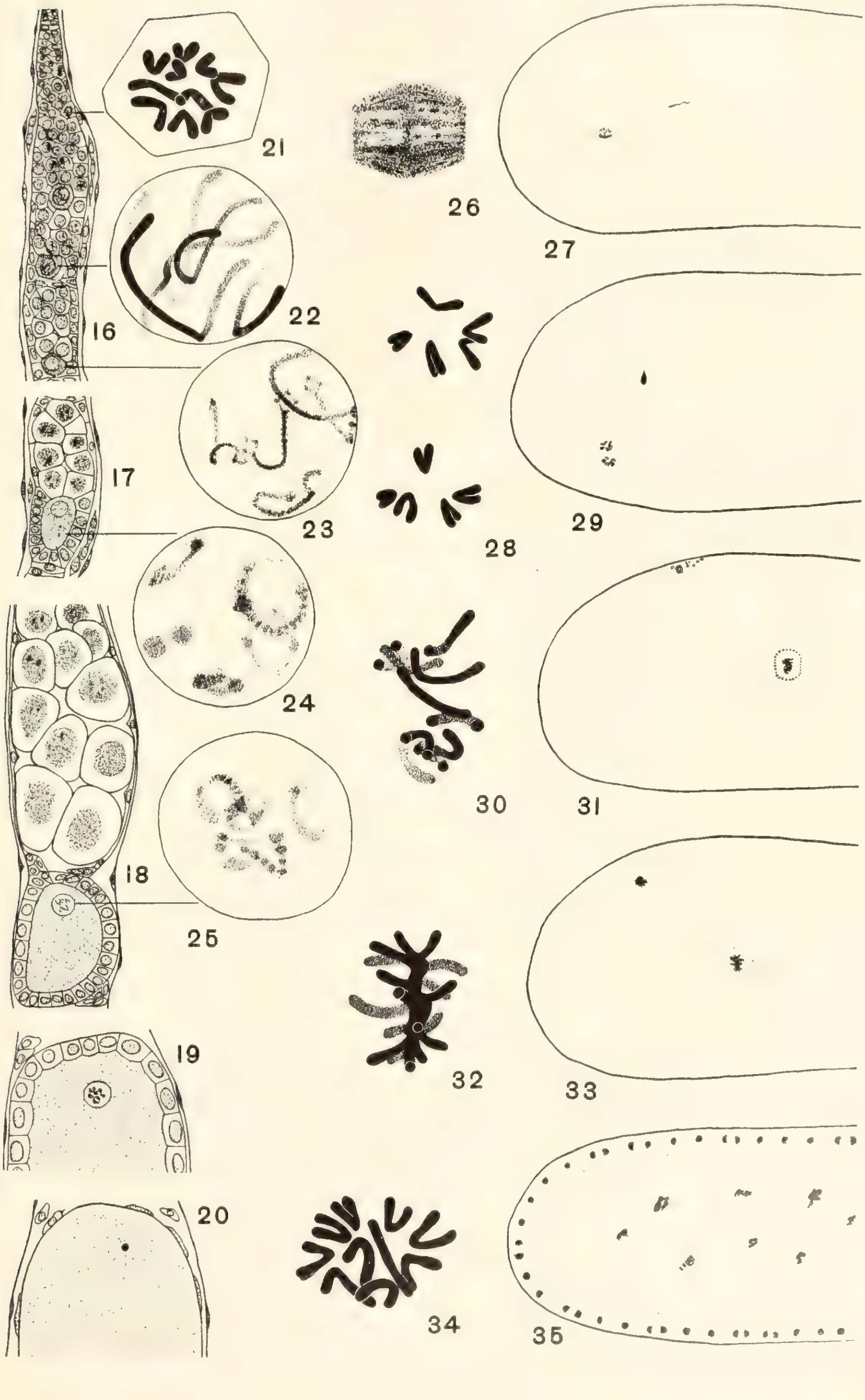
FIG. 32. First cleavage metaphase of a fertilized egg, from Fig. 33.

FIG. 33. Egg in metaphase of first cleavage; the polocytes, optically superposed, are at the surface.

FIG. 34. A metaphase from the blastoderm; from caudal end of egg in Fig. 35.

FIG. 35. Cephalic end of egg with young blastoderm; the yolk nuclei are in the center; the blastoderm cells, in this region, are all in anaphase.

PLATE II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The 5 chromosomes of the second oöcyte and those of the first polocyte are also present and are distinguishable by their relative positions, the oöcyte chromosomes being nearer the center of the egg while those of the polocyte are near the surface. Both are shown on a larger scale in Fig. 11.

After completion of the second oöcyte division, normal, haploid cleavage may set in in a small percentage of the eggs; but it is not at present known whether such normal cleavage can actually occur in eggs containing a foreign sperm or whether it is confined to eggs which may have escaped penetration by a sperm. In any case, whenever cleavage proceeds normally the nuclei are invariably haploid. The first cleavage mitosis occurs in the center of the anterior half of the egg. During the succeeding cleavages, the mitotic stages are strictly synchronous up to the time of formation of the complete superficial blastoderm, 12 hours later, when the divisions at the hind end of the egg begin to lag slightly behind those at the anterior end. Figure 15 represents the anterior end of an egg in which the cells are all in metaphase of the eighth cleavage division. Polar and lateral views of typical cells from this egg are also shown on a larger scale (Figs. 13 and 14), with the slender, elongated chromosomes characteristic of these divisions. In germ-band stages, in late embryos and in young larvæ hatching from such eggs only the haploid number of chromosomes has ever been found.

Development of Fertilized Eggs

In normally fertilized eggs the meiotic stages are like those just described for unfertilized eggs. The sperm is most often found at first caudad to the oöcyte nucleus whence it proceeds to the region where the first cleavage mitosis is to occur. Of the oötids, the one which is to take part in syngamy also approaches this region, while the polocytes come to lie at the periphery of the egg. In an egg less than four minutes old (Fig. 27), the sperm head is slightly swollen and the oöcyte nucleus (shown on a larger scale in Fig. 26), now shaped like a truncated spindle, is also swollen and is approaching the periphery. Figure 29 shows the end of the first meiotic division as well as the enlarged sperm; and Fig. 28, the two chromosome groups more highly magnified. The prophase of the first cleavage mitosis (Figs. 30 and 31) contains 10 chromosomes and it is possible to distinguish two groups of five chromosomes each, one group derived from the male pronucleus, the other from the female. This stage is followed by a metaphase (Figs. 32 and 33), of which only the lateral view is obtainable in an egg which has been mounted entire. The succeeding cleavage divisions and the later stages of development are similar to those described above for unfertilized eggs, excepting that the diploid number of chromosomes is present. Figure 35 represents an egg in which the cleavage nuclei have just entered the periplasm to form the completed blastoderm. Embedded in the yolk are a number of chromatic masses, the yolk nuclei, while at the surface, each blastoderm cell is in anaphase, excepting those cells at the extreme caudal end of the egg which are still in metaphase. One of these metaphases is shown on a larger scale in Fig. 34.

DISCUSSION

It is apparent that the males in this species are haploid and develop from unfertilized eggs of either mated or virgin females, while the females are always diploid zygotes. Sex determination thus appears to follow the Dzierzon Law except that, in *Melittobia*, haploidy generally has a lethal effect and only a small proportion of the unfertilized eggs is capable of development.

There is nothing, however, in our data that would exclude the validity of the Whiting theory of sex determination. In attempting to apply the latter theory here, we should have to note again that haploidy is lethal in most eggs and that there is as yet no evidence for the survival of zygotes produced by homeosyngamy, i.e., for biparental males.

Biparental males may be looked for only among the offspring of normally mated females. Not more than four such males were examined cytologically. The remainder of the males examined were all obtained from virgins or from females mated with males of a different species. Although the direct evidence against the occurrence of biparental males is inadequate, the fact that the number of eggs yielding males, compared with the total number of eggs laid, is no larger (ca. 3 per cent) in females normally mated than in females not so mated would seem to suggest that biparental males are not produced. We may therefore conclude that homeosyngamy, if it occurs, is lethal. Without the presence of distinguishable X and Y chromosomes the existence of invariably lethal homeosyngamous combinations is not demonstrable. If such combinations do occur, we should expect to encounter a corresponding number of inviable eggs in the layings of normally mated females. This one condition, at least, is actually met. There is normally a considerable egg mortality observed, which seems to vary in different individuals. In the only instance in which an accurate count was made it amounted to 21 per cent out of the first 224 eggs laid.

The fact that the ratio of males to females (3 : 97), produced by normally mated females, is approximately the same as the ratio of males to unhatchable eggs produced by females not so mated, brings to mind an idea expressed by Lenhossék and Godlewski. As expressed by Godlewski, this idea is that in honey bees two sorts of eggs are produced, of which only the one sort, the female-producing, is capable of and requires fertilization, while the other produces males parthenogenetically. Although it is now generally recognized that in honey bees every egg is capable both of parthenogenesis and of fertilization, the evidence in *Melittobia* appears to favor Lenhossék's idea. However, unless we can state more definitely the nature of the differences between the postulated two sorts of eggs, any hypothesis based on this idea remains rather gratuitous and does not contribute to any real understanding of the mechanism responsible for the unusual sex-ratio and the related reproductive phenomena observed in this species. In the material examined there have not been recognized, as yet, any visible differences in the chromosomal behavior during oögenesis or later, which might be considered responsible for the failure of haploid development in most eggs and its success in some.

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THE RELATION OF FAT CHANGES TO THE GENERAL CHEMICAL EMBRYOLOGY OF THE SEA URCHIN

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INTRODUCTION

Several years ago (Hayes, 1934) a study was made of the nitrogen metabolism in the early egg stages of the Florida sea urchin, *Echinometra lucunter*. In the previous year Ephrussi published a general account of the metabolism of developing *Paracentrotus* eggs. The papers agreed in finding a loss of nitrogen during later development; for the period immediately following fertilization there are no data in the latter paper, and in the former the results suggested an increase in nitrogen which, since such a state of affairs is otherwise unknown in embryology, was treated with caution pending confirmation. Nitrogen then, and therefore protein, is evidently a source of energy in later development, probably from the eight-hour hatching stage on.

This paper records the results of a study of another possible source of developmental energy, namely fat. Ephrussi (1933) offers the only earlier study, and he found a diminution of fat as development proceeded. Since he tested only three ages, namely unfertilized, 12 hours and 40 hours, his results do not show the precise time at which the loss took place, and cannot be used to supplement and check those to be presented below.

Ephrussi expresses his results as percentages of the wet or dry weight of the egg, a procedure which can be standardized when one is dealing with unfertilized eggs alone, but which presents great difficulties when it is necessary to take into consideration the changes in volume and shape which occur during development. Eggs usually have to be concentrated for analysis by centrifugation, and the number of eggs which will occupy 1 ml. in a centrifuge tube at a given speed is not the same at any two ages. If eggs are sectioned and examined it is possible to derive formulae for the estimation of volume at different stages (Pelluet, 1938). If the volumes are now combined with counts showing how many eggs can be packed into 1 ml., data are at hand for the expression of any egg constituent as a percentage, e.g. fat. This procedure would be very tedious; has not been carried out by any investigator; and if it were would not be profitable because the truth would be obscured by fluctuations in the water content which would make e.g. a stationary fat concentration per egg appear to vary. For these reasons and others which have been discussed at length (Hayes, 1934), the values below are given per million eggs rather than in per cent.

METHODS

The first requirement in work of this kind is a method for rearing eggs in large numbers, free from debris and bacteria, and showing a high percentage of normal development. Attempts were made at first to work with starfish eggs, but the last condition could not be satisfactorily met, and *Arbacia* was substituted. Eggs were obtained from several females, fertilized and reared in finger bowls according to the well-known methods devised by Just and others. All stages were examined to ascertain the percentage developing normally and it was always over 90.

When it was desired to collect a sample the eggs were concentrated in centrifuge tubes at low speeds. This procedure could not be used for swimming stages, which were collected by filtration through fine bolting silk. Following either treatment, eggs or larvae were transferred to a volumetric flask of sea water, usually of one liter capacity, and the water made up to the mark. Water and eggs were then transferred to a larger vessel and thoroughly mixed. While the mixing was continued 1 ml. samples were withdrawn with a brass Stempel pipette and transferred to a Sedgwick-Rafter plankton counting slide of capacity 1 ml., provided with a cover slip. (I am indebted to Dr. C. J. Fish for the loan of a Stempel pipette and counting slide.) Ten fields of the slide, selected at random, were then counted with a microscope whose lenses had previously been calibrated with a stage micrometer. The total was added up and constituted one observation. Usually four such observations were made on each of two samples and the results averaged. Two observers alternated on the counting in order to eliminate the personal factor as far as possible. The probable errors in counting ranged from ± 0.39 per cent to ± 10.2 per cent with an average value of ± 4.1 per cent.

After the counts were made the eggs were again concentrated in centrifuge tubes, and transferred from these to a 100 ml. volumetric flask. The fats were extracted by the wet alcohol-ether method described by Fowweather (1926), the flask was made up to the mark, and the fat-containing extract filtered off and stored in glass-stoppered bottles, evaporation being prevented by sealing the tops of the bottles with several coats of silica (water glass). When the analyses were made some months later, portions of the alcohol-ether extract were dried in a partial vacuum at room temperature and re-extracted with petroleum ether. These were made up to known volume and stored in cork-stoppered bottles, sealed by silica.

The total fat or phospholipid or sterol digitonide, was estimated by a micro-method devised by Backlin in 1930, described by Peters and Van Slyke in 1932, and slightly modified by Van Slyke, Page and Kirk (1933). Carbon dioxide, produced by the combustion of a fat sample in a special test tube attached to a Van Slyke volumetric or manometric apparatus, is carried over and reacts with dilute NaOH in the chamber, being subsequently liberated by the addition of excess lactic acid, and measured as a gas. The probable errors resulting from extractions and fat determinations are less than ± 2 per cent. For conversion of the CO_2 into fat or into sterol the factors are given in Peters and Van Slyke, page 437. A similar table was constructed for phospholipids, based on data in Bloor (1929).

TABLE I

Variations during development in total fat, sterol and phospholipid per million eggs.

1 Age in hours and minutes	2 Total fat, mg. per million	3 Sterol, mg. per million	4 Phospholipid, mg. per million
Unfert.	5.65	0.430	2.57
1.	3.98	0.413	2.53
4.40	3.50	—	—
6.30	3.36	0.413	3.3
8.50	3.04	—	0.81
10.50	3.60	—	2.04
15.35	3.80	—	2.48
19.30	3.92	0.431	—
23.15	3.84	—	3.46
24.50	4.29	—	—
25.25	3.25	—	1.2
43.10	2.11	0.416	—

Sterol was precipitated with digitonin directly from an aliquot of the original alcohol-ether extract in one of the combustion tubes. The precipitate was washed with ether and with water, supernatent fluid being removed by means of suction through an alundum filter stick as described by Kirk, Page and Van Slyke (1934). After drying, the sterol digitonide was oxidized to CO_2 as described above. The method was checked and found to give theoretical values with cholesterol.

TOTAL FAT

By total fat is meant all the material soluble in petroleum ether. Its variations are given in Table I, column 2 and in Fig. 1. It will be noted that from an initial value of 5.65 mg. per million in the unfertilized egg, there is a steady drop until by 9 hours there are only 3.04 mg. From this time until 20–25 hours an increase is noted, followed by a drop to the final stage observed. The most interesting points are that the trough of the curve coincides with the time of hatching, and that there is evidence of fat synthesis from approximately 9 to 23 hours. The shedding of the shell and the beginning of fat synthesis may well be associated with one another. No previous reference to the manufacture of fat by invertebrates has been found, although the phenomenon is not unknown in teleost embryos (Hayes and Ross, 1936). Since the embryo has at this time no organs of digestion, the source of the new fat is presumably some other material in the egg. There is no proof as to what this is, but a hint is obtained from the nitrogen metabolism of the Florida sea urchin, *Echinometra* (Hayes, 1934), in which, at about the time of hatching a decline in the non-amino nitrogen began, which was practically arrested when the observations closed at 24 hours. (The amino nitrogen, on the other hand, did not begin to decline until the sixteenth hour.)

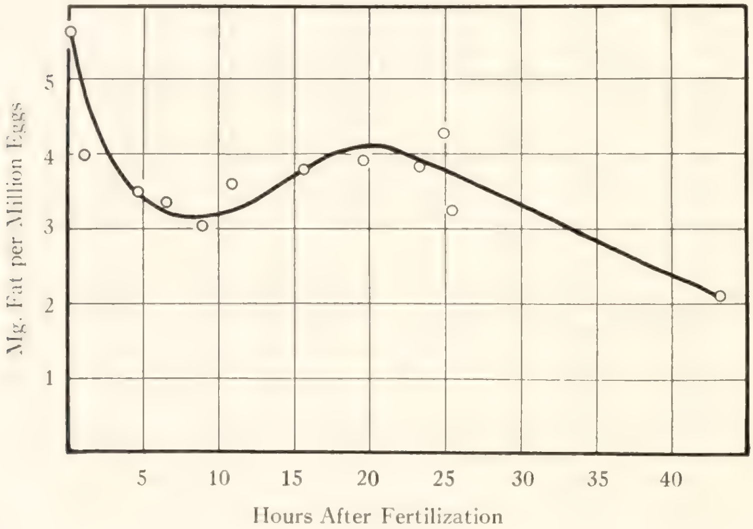

FIG. 1. Fluctuations in total fat during development. Note that the time of hatching, 8 hours, corresponds to the initial trough of the curve.

Ephrussi's three values for total fat were as follows, given in each case as percentage of the wet weight.

Unfertilized	4.81
12 hours	4.43
40 hours	3.69

A glance at Fig. 1 will show that the peak at 20 hours will be missed by the particular stages selected by Ephrussi, so that his gentle decline is in agreement with Fig. 1.

Three further pieces of evidence may be mentioned, after which it will be possible to present a provisional picture of the general chemistry of early development. Warburg (1915) found in *Strongylocentrotus* a sudden burst of respiratory activity at fertilization, followed by a steady increase as development proceeded. The respiratory quotient over the first three hours was 0.9.

Secondly Runnström (1933) observed in *Paracentrotus* a sudden production of acid at fertilization. It was not carbonic acid—in fact the pH of the egg was altered to such an extent that considerable quantities of CO_2 were driven off. The consequence was that Runnström, who was making an investigation of the changes in respiration during the first hour, found that the excess CO_2 caused the apparent R.Q. for the first 10 minutes to soar to 2.37. Within half an hour the mysterious acid was being used up, and respiratory CO_2 consequently held in the egg as bicarbonate, with the result that the apparent R.Q. for 30–45 minutes was only 0.45. Although Runnström did not discover what the acid in question was, he showed that it was not phosphoric acid, and he cited the work of Perlzweig and Barron on *Arbacia* to prove that it was not lactic acid. The inference is that carbohydrate metabolism is not responsible. It may be noted here for future reference that Page (1927) found that the oil of *Arbacia* eggs had a saponification value of 606; of *Asterias* eggs 319. The oils also yielded large quantities of volatile fatty acid when distilled with steam. Fatty acids from such oils would have a low molecular weight, high solubility in water, and adequate strength to drive out CO_2.

Finally, evidence has been accumulating for some years which suggests that two kinds of respiration go on simultaneously in the egg (see Loeb and Wasteneys, 1911). It is not improbable that two substrates are burnt and that two enzymes facilitate the oxidations. That one of these belongs to the general group of oxidases, peroxidases and catalase is shown by the fact that the respiration both of fertilized and unfertilized eggs is stimulated by dimethylparaphenylene diamine (Örström, 1932). This reagent is absorbed by the eggs and becomes in effect an artificial substrate, the measure of whose oxygen requirement is an index of the available excess enzyme in the egg. The enzyme system then, is present before fertilization, but of course the amount of oxidation which it can bring about is strictly limited by the quantity of available substrate. A second characteristic of the oxidase type of enzyme is its susceptibility to cyanide poisoning. Now cyanide has very little effect on the respiration of the unfertilized egg (Runnström, 1930), but it produces a marked inhibition of oxygen consumption after fertilization. The conclusion is that there is an abundance of enzyme, but practically no substrate before fertilization, and a small but steady supply after fertilization, which is used up as produced. As to the second part of respiration (not inhibited by cyanide) it was found that it could be stopped by narcotics, e.g. lithium (Lindahl, 1934), and stimulated by pyocyanine (Runnström, 1935*a*, both fertilized and unfertilized eggs), and by methylene blue (tried on unfertilized eggs only by Barron, 1929). Thus it passed the tests for a dehydrogenase system. Pyocyanine acts as an artificial enzyme, and the stimulus caused by its presence meant that there must have been some extra substrate there to be burnt up; in other words (in contrast with the first part) the substrate was plentiful and the small quantity of enzyme was the limiting factor.

Taking all these facts into consideration, assuming that the various sea urchins resemble one another in the essential features of their embryonic metabolism, and remembering that the times of hatching approximately coincide, the following general hypothesis of echinoid chemical embryology may be advanced.

(1) At the time of fertilization a considerable quantity of fat is split into fatty acid and glycerol, presumably by means of a lipase, the activation of which is the first of the chain of events initiating embryonic metabolism. It would be tempting to believe that the lipase is brought in by the spermatozoön, but no evidence has been found bearing on this point. For every molecule of glycerol produced there are three molecules of fatty acid; and for every calorie of energy available from glycerol there are 5 or 10 calories available from a lower fatty acid. The glycerol is scarce, the acid plentiful.

(2) The glycerol is rapidly oxidized by a cyanide-sensitive enzyme. This part of respiration does not increase as development proceeds. The enzyme was there before fertilization, but was inactive because of the absence of suitable substrate. The destruction of glycerol accounts in part for the increase of activity found at fertilization by Warburg. Glycerol continues to be produced in small quantities up to at least 8 hours, but is oxidized as formed. That cell division is

the part of development presided over by cyanide-sensitive respiration was shown many years ago by Loeb and Wasteneys (1911), who found that the concentration of cyanide exactly necessary to block development reduced the oxygen consumption to one-quarter its normal value, an amount which coincided with the normal requirement of unfertilized eggs. Runnström (1935*a*) has recently confirmed this experiment, and extended it by immersing eggs in a mixture of cyanide and pyocyanine at the same time, thereby inhibiting one part of respiration and stimulating the other. The net result was an increase in oxygen consumption, but a blocking of cell division in early prophase.

(3) Runnström's unknown acid is fatty acid. It is oxidized by the second, or cyanide-insensitive dehydrogenase system. The activities resulting from this energy source are presumably growth and basal metabolism. The enzyme does not oxidize all its substrate at once—if it did the egg would burn out in a very short time. Nevertheless, this part of respiration is shown by experiment to increase in intensity as development proceeds. An explanation of the paradox of how a limited quantity of enzyme can be made to do more work later in development than at the beginning is obvious from the work of Spek (1934), who showed that in *Asterias* and *Arbacia* eggs acid is not distributed evenly throughout the whole egg, but is strictly localised. Before fertilization the surface is acid, the interior alkaline; in early developmental stages there is a thin acid layer surrounding the dividing cells, and from the 64-cell stage to the gastrula there is a gradient of intensity of acid reaction between the animal and vegetal poles. The chemical geography of the egg limits the extent to which enzyme and substrate are able to come together. This conception is strengthened by Runnström's (1935*b*) statement that "All our experimental evidence indicates that the susceptibility to the action of lithium is highest at the animal pole and decreases gradually."

(4) Fat ceases to be a source of energy at approximately the time of hatching, 8 hours, possibly because it is required as building material.

(5) From hatching time up to 16 hours energy is provided by non-amino nitrogenous materials (non-protein?), from which fat is synthesized and possibly the costs of cell division, motion and basal metabolism are in part or in whole met.

(6) To the utilization of non-amino nitrogenous materials there is added at 16 hours, a gradual destruction of compounds containing amino nitrogen as well.

(7) At 24 hours fat begins once again to decline while at the same time the destruction of nitrogenous material appears to be concluding.

(8) Ephrussi (1933) reports the loss of almost all the carbohydrate in the egg between his 12- and 40-hour tests, and very little loss in the first 12 hours. This suggests that carbohydrate is the fuel source drawn upon in later stages, possibly by about 24 hours. The embryo is perhaps using up its last reserves of food and consequently entering upon a period of starvation, pending the development of its digestive system to a point where food can be taken from the sea.

(9) Hence the apparent sources of energy in succession are: fat, non-animo nitrogen, amino nitrogen, fat, carbohydrate. More detailed information may move carbohydrate to the second or third last place.

The assignment made above of cyanide-sensitive enzyme to glycerol (or a product of it), and of cyanide-insensitive enzyme to fatty acid, is based not only on the relative abundance of the substrates, but also on the work of Emerson (1927), which indicates that cyanide-sensitive respiration results from the oxidation of carbohydrate.

Lindahl and Öhman (1936) have recently placed on some of the results cited above an interpretation differing from that given here. They say, "We see a useful conception of these relationships in the assumption that two different substrates are burnt, of which one has a small, the other a high degree of reactivity to the oxidizing 'carrier.' Shortly after fertilization only the first substrate is present in large quantities. The unchanging and small concentration of the 'carrier' determines the constant and small intensity of oxidation of this substrate. This reaction corresponds to the non-growing part of respiration, which can be increased to a marked degree by dimethylparaphenylene diamine and pyocyanine. The 'carrier' is here the limiting factor. The other substrate is very rapidly oxidized and hence does not accumulate. The rapidity of formation of this substrate determines the oxygen consumption, and is therefore the limiting factor. This is the 'growing part' of respiration, which can be inhibited by lithium." It will be seen that Lindahl and Öhman differ from the views expressed in this paper in that they: (*a*) postulate two substrates but only one enzyme system; (*b*) express no view as to the nature of the substrates; (*c*) consider that dimethylparaphenylene diamine and pyocyanine act on the same part of respiration; (*d*) state that the cyanide-sensitive part of respiration is limited by the low degree of reactivity of the substrate (and not by its scarcity); (*e*) state that the cyanide-insensitive part of respiration is limited by the scarcity of the substrate (and not by geographical segregation).

STEROL

The values for sterol will be found in column 3 of Table I. It amounts to some 0.41–0.43 mg. per million eggs, and no fluctuation was found in its concentration during the period investigated. The only comment necessary is that this particular one-tenth of the fat is not a source of embryonic energy.

PHOSPHOLIPID

The results of phospholipid determinations are given in column 4. They vary from less than 1 mg. to nearly 3.5 mg. per million eggs. The values unfortunately fluctuate so much that attempts to discover a developmental trend by plotting them on a graph are unconvincing. No utilization of phospholipid can be said to be demonstrated, although the data are not sufficiently extensive to exclude the possibility. The average of all the phospholipid results is 2.17 ± 0.24 mg. per million eggs or 38 ± 4 per cent of the total fat in the unfertilized egg.

PREVIOUS ANALYSES

McClendon (1909) found that the total fat (ether extract) of unfertilized *Arbacia* eggs was 2.254 per cent. Harvey (1932) gives the data necessary to convert this to mg. per million as follows:

$$\text{Mg. per million eggs} = \frac{10^6 \times \text{vol. of 1 egg in cu. mm.} \times \text{egg density} \times 2.254}{100}$$
$$= 2.12 \times 1.09 \times 2.254$$
$$= 5.21 \text{ mg.}$$

McClendon also estimated the phosphorus content of his ether extract and found it to be 0.06914 per cent of the whole egg. Assuming that all the phosphorus is in the phospholipids and that it comprises 4 per cent of these (as it does in lecithin) then this figure too can be made comparable.

$$\frac{2.12 \times 1.09 \times 0.06914 \times 100}{4} = 3.84 \text{ mg. phospholipids per million eggs.}$$

If it is valid to apply these calculations to McClendon's results then it follows that they agree in a general way with those given above.

Page (1927) has also made analyses of the fatty constituents of the unfertilized *Arbacia* egg. He states that 8.3 grams of oil were obtained from 183 million eggs by means of alcohol-ether extraction. This is in the terminology of this paper, 45.4 mg. per million eggs, or ten times as great as McClendon's figures and those given here. A calculation of the mass of an egg from the volume and density values in Harvey shows that, according to Page, 20 per cent of the egg is fat. Now since the total solids (McClendon) only comprise 20 per cent of the egg and these are obviously not all fat, Page's results are difficult to interpret, particularly in view of his statement that *Arbacia* contains much less oil then *Asterias*. Page also precipitated the phospholipid from his extract with acetone, and his results work out at 8.4 mg. per million as against McClendon's 3.84 mg. and the 2.17 mg. above. Finally Page distilled the volatile fatty acids from his extract with steam and titrated them with NaOH, finding 9.4 mg. per million eggs. The discrepancy between Page's results and the others may lie in part in his method of counting eggs, no mention of which is made in his paper. This difficulty would not invalidate his estimates of the saponification value and his volatile acid determinations on which a part of the general argument in this paper depends.

Summary

Periodical estimations were made of the total fat, sterol and phospholipid during the first 40 hours of development of *Arbacia*. The total fat decreases up to the time of hatching (8 hours), then increases for some 10 hours, and later decreases again. The sterol concentration remains unchanged throughout the period studied. Owing to fluctuations in the phospholipid readings, a definite conclusion could not be drawn as to whether this material is utilized as a source of embryonic energy. The total fat in a million unfertilized eggs is 5.65 mg. of which 7.5 per cent is sterol and 38 per cent phospholipid. A general hypothesis of the chemistry of early sea urchin development is presented.

It is a pleasure to acknowledge my indebtedness to Professor E. G. Young, whose advice had been frequently sought and most generously given.

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ON THE SPECIFICITY AND RELATED PROPERTIES OF THE CRUSTACEAN CHROMATOPHOROTROPIC HORMONE

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INTRODUCTION

When the eye-stalks of several brachyuran crustaceans are removed, the animals become pale within two hours (1), (2), (3), (4). Injection of a sea-water extract of the extirpated eye-stalks into the blinded (eye-stalk-amputated) specimens results in the appearance of the dark coloration (3), (5). Although several kinds of chromatophores are involved in these color changes, the movements of the melanophores are chiefly responsible for the resulting external coloration, and in the following experiments were the only chromatophores studied in the fiddler crab, *Uca pugilator*.

RESULTS

Method of Determining the Relative Activity of Eye-stalk Extracts

A new method of assaying the eye-stalk hormone was devised so that determinations could be made within an hour. The techniques of preparing the hormone, injecting the animals, and preparing the animals for the test were essentially the same as described previously (5). The plan of the method was as follows: Each of 8 groups of 15 blinded animals per group was injected with one of 8 different, known concentrations of hormone (1.0 E.S.[1]–0.005 E.S. per cc. of solution). The percentage of animals showing the slightest perceptible response (melanophore stellation) was determined at 5-minute intervals following the time of injection for a period of one hour. If the percentage of animals showing the slightest perceptible response at various concentrations is plotted against time, a series of steep, sigmoid curves is obtained whose inflection points intersect the ordinate at about 50 per cent. The times at which 50 per cent of the animals show the response at different concentrations range from 8 minutes for the strongest to 24 minutes for the weakest. A standard curve (Fig. 1) was then constructed, using a range of concentrations from 0.04

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[1] The letters E.S. are the abbreviation for eye-stalk. The letters E.S.H. will be used as the abbreviation of eye-stalk hormone.

E.S./cc.–0.002 E.S./cc. Figure 1 can, therefore, be utilized for determining the relative strength of very dilute solutions of the hormone. For stronger concentrations (1.0 E.S./cc.–0.03 E.S./cc.) Fig. 2 was employed, indicating the relationship between concentration and percentage of animals showing the full response (complete melanophore expansion) at 1 hour following the time of injection. For still stronger solutions, the method previously described (5) was employed. By using Figs. 1 and 2 as characteristic curves, the relative strength of an unknown concentration can be determined by taking a reading, at 30 minutes after injection, of the percentage of animals showing the slightest perceptible response, and another at 1 hour giving the percentage showing the full response.

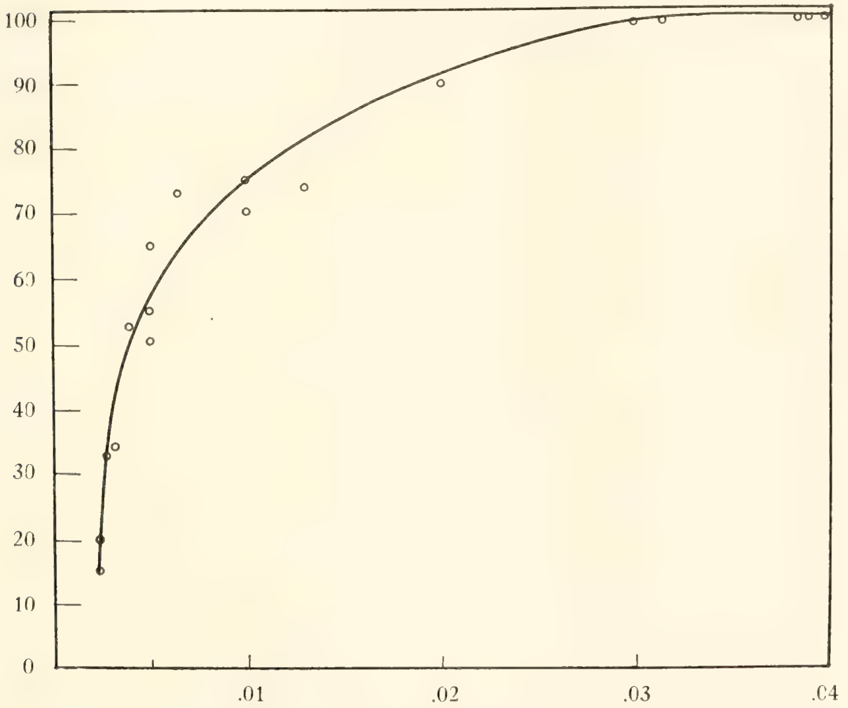

FIG. 1. Curve showing relationship between percentage of animals showing the slightest perceptible response (melanophore stellation) and concentration. Ordinate—percentage of animals responding; abscissa—concentration expressed as eye-stalks per cc. of solution.

A study of these and other curves not included in the text reveals several points of interest concerning the nature of melanophore response in *Uca*. From the results shown by Fig. 1, where very small amounts of hormone were injected, it is evident that a certain threshold must be reached before the melanophores react and, moreover, that this threshold varies from animal to animal. With stronger doses, the degree and the duration of melanophore expansion are exponentially proportional to the dosage injected. Thus, melanophores, like certain types of smooth muscle, show graded responses whose magnitude varies as the strength of the stimulus. For a complete expression among time, concentration of hormone, and the various phases of melano-

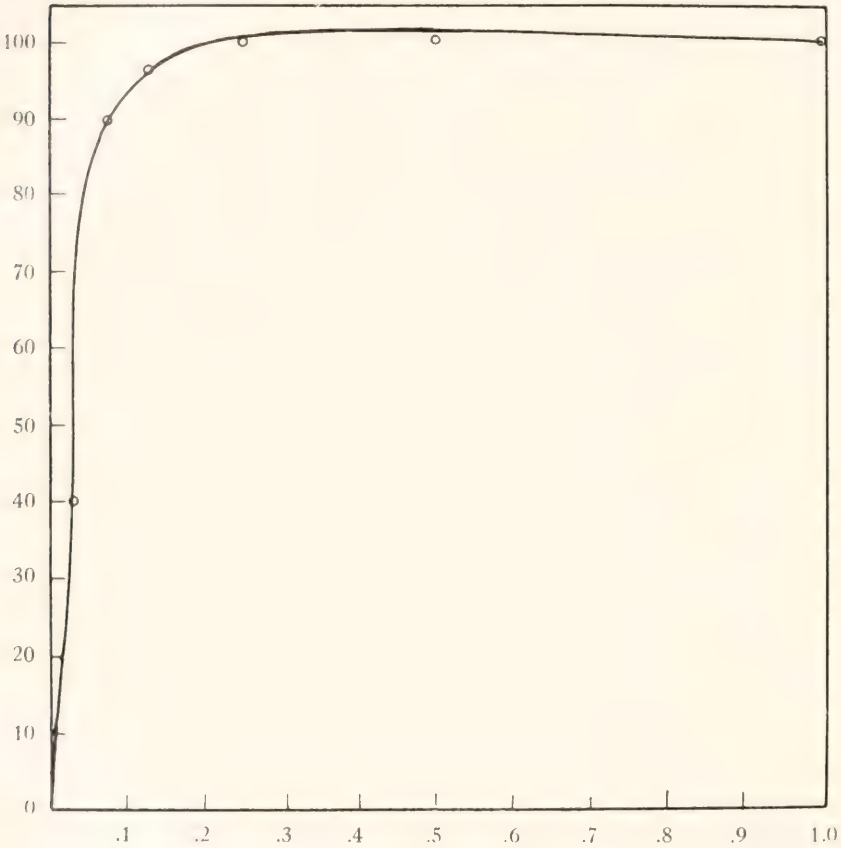

FIG. 2. Curve showing the relationship between percentage of animals showing the full response (complete melanophore expansion) and concentration of hormone. Ordinate percentage of animals showing full response; abscissa concentration expressed as eye-stalks per cc. of solution.

phore response, the construction of several nomograms is necessary. The curves already presented, however, suffice for the present studies.

Sensitivity of the Test; Estimation of the Amount of Hormone in the Eye-stalk

The strength of eye-stalk extracts, as read off from the curves, is not constant from animal to animal. Those from larger animals (5.0 grams) usually assay at higher values, although there is always some discrepancy in a group of the same size and sex. The sensitivity of the test may be illustrated by the following experiments: a sea-water extract of 1 eye-stalk (animal weight = 5.0 grams) was diluted until the total volume was 400 cc. Sixty-six per cent of the animals injected with 1/20 cc. of this solution responded. When further diluted to 600 cc., 50 per cent responded; to 800 cc., 33 per cent responded. With still greater dilutions, the percentage quickly fell to zero. We take the point at which 50 per cent of the animals respond as a reliable indication that an extract is active, and in the following discussion, we assume this point (50 per cent response) to indicate a minimal unit of activity, which is 1/600 of a *Uca* unit (5).

The dry or nearly dry weight of an eye-stalk is 2.0 mg. (average of 600 eye-stalks). From microscopic sections of the eye-stalks of *Uca pugilator*, we estimate the sinus gland of Hanström (6) to be, roughly, 1/100 that of the eye-stalk. Assuming the proportion of the active principle to be 1/100 that of the gland, which is probably a conservative surmise, the total amount of hormone in one eye-stalk is therefore about 0.2 γ. The sensitivity of this biological test is such that 0.2 $\div$ 600 $\times$ 20 (since each animal is injected with 0.05 cc.) or 0.000016 γ of hormone can be detected. These figures, of course, are very rough but, we think, indicate the probable order of magnitude.

Specificity of Action

Thus far, extracts of only two other glands, the pituitary (7) and the subneural gland complex (8), have been found to produce melanophore expansion when injected into blinded *Uca*. We found accidentally that the injection of distilled water produces strong melanophore responses. In 55 crabs, blinded for from 2–8 days previously, the injection of distilled water produced in 15–20 minutes complete melanophore expansion in 90 per cent of the animals. After 1 hour, 50 per cent became pale, and after 2 hours, 90 per cent–100 per cent became pale. Quantitatively, this effect is quite different from that produced by the eye-stalk hormone in any concentration. Injection of distilled water into 25 normal dark animals was without effect.

Since this reaction is of interest in relation to the general problem of hormone specificity, we examined several possibilities which might explain the effectiveness of distilled water. It is quite certain that injection of sea water does not produce any responses. Only 1 of 46 blinded animals injected with sea water responded weakly but positively, while no significant changes were detected in 10 normal dark animals also injected with sea water. Sea water, therefore, must be employed as the solvent when testing preparations of E.S.H. Since distilled water is acidic (pH 5.7) as compared with sea water (pH 7.7), we injected tap water (pH 7.0) into 10 blinded and 10 normal *Uca*, with results identical to those already described for distilled water. Identical results were also obtained with distilled water made alkaline (pH 8.0) by the addition of 0.01 N NaOH. The acidity of distilled water is therefore not responsible for its chromatophorotropic activity.[2]

Another difference between the action of the hormone and that of distilled water was obtained by injecting various dilutions of sea water with distilled water. The following proportions of sea and distilled water were tried:

Sea Water		Distilled Water	Chromatophorotropic Activity
1 part	plus	1 part	Inactive
1 part	plus	2 parts	60 per cent of animals respond
1 part	plus	3 parts	80 per cent of animals respond
1 part	plus	6 parts	80–100 per cent of animals respond
1 part	plus	10 parts	80–100 per cent of animals respond
1 part	plus	16 parts	80–100 per cent of animals respond

Since the injection of 0.05 cc. distilled water must dilute the blood of a crab appreciably, osmotic effects were studied. Assuming the freezing point depression of *Uca* blood to be close to that of lobster blood, we injected sucrose (1.3 M) isosmotic with the latter into 20 blinded crabs. No melanophore expansion resulted. Sucrose (0.95 M) isosmotic with sea water, however, produced a slight reaction in 20 per cent of the animals. It seemed, therefore, that the effect of distilled water may be due to the resulting hypotonic condition of the blood. The following salts isosmotic with sea water were also tested:

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[2] The pH of distilled, tap, and sea water at Woods Hole was determined by means of the glass electrode.

		Blinded Specimens (20 animals per test)	
Salt		General Effects	Chromatophorotropic Activity (per cent of animals responding)
NaCl	0.52 M	—	30
$MgCl_2$	0.51 M	—	20
$CaCl_2$	0.34 M	—	20
KCl	0.53 M	Muscular twitchings and death	0
Na_2HSO_4	0.4 M	Immediate prostration, death after 1 hour	10
LiCl	0.52 M	Immediate twitchings of legs	20
200 per cent sea water		—	0

While the effect of drugs on vertebrate melanophores has been determined, no similar investigation has been made on crustacean melanophores. Sixteen different, chemically-pure drugs were injected into both normal and blinded crabs. One hundred γ of the drugs, dissolved in sea water, was injected into each of 10–20 animals of both groups. Several drugs produced violent, convulsive movements, prostration or twitchings of the legs while others were entirely without muscular effects. Table I summarizes the action of 100 γ of the drugs:

TABLE I

Effects of sixteen drugs on normal and blinded crabs

Drug	Blinded (Pale Animals)		Normal (Dark Animals)	
	General Effect	Chromatophorotropic Activity (Melanophore expansion)	General Effect	Chromatophorotropic Activity (Melanophore contraction)
Atropine sulphate	—	—	—	—
Morphine sulphate	—	—	—	—
Acetyl choline	—	20% (slight)	Shedding of legs	—
Histamine	Paralysis of legs	—	Slight paralysis	—
Eserine sulphate	Prostration and death	—	Not performed	
Pilocarpine HCl	—	—	—	—
Cocaine HCl	Rigidity	30% (slight)	Rigidity	—
Brucine sulphate	Paralysis	—	Sluggishness	—
Veratrine sulphate	Instantaneous death	—	Instantaneous death	50% slightly pale
Curare	Paralysis	50% (slight)	Paralysis	—
Strychnine SO_4	Temporary paralysis	20% (slight)	Paralysis	—
Guanidine	Prostration	20% (slight)	—	—
Chlorbutanol	—	20% (slight)	—	—
Caffein	—	20% (slight)	—	—
Nicotine	Prostration	40% (slight)	Prostration	—
Hyoscine HBr	—	80% positive	—	—

None of these drugs with the exception of hyoscine hydrobromide produced definite, positive results in blinded specimens, and all were without effect on the melanophores in normal dark specimens. These drugs (with the one exception) have therefore no direct or indirect action on contracted or expanded melanophores. We did not repeat these injections in normal animals during the night (nocturnal or pale phase) although possibly some of these drugs may effect a release of the hormone from the sinus gland, which appears to be under nervous control (5).

Adrenalin, practically universally, produces melanophore contraction in vertebrates in extremely small doses. Adrenalin, in various dilutions from 1 : 1,000 to 1 : 10,000 (dilutions greater than 1 : 1,000 being made with sea water) was without significant melanophore responses when injected into either normal or blinded specimens. Strong doses usually produced death in normal animals, while 20 per cent of the blinded animals showed slight melanophore stellation. The activity of adrenalin was confirmed by injecting 0.2 cc. of each dilution subcutaneously in normal dark catfishes. This experiment illustrates effectively the point made elsewhere (4) that melanophores of various animals do not always react in the same way to the same substance, for example, chemically pure adrenalin hydrochloride. It is only logical to think of a response to a hormone in terms of the interaction between the responding tissue and the hormone in question, not solely in terms of the hormone itself. If the same type of responding tissue in two different animals is physiologically and anatomically different, as are the melanophores of crustaceans and vertebrates, it is not surprising that they react in different ways to the same substance. The ineffectiveness of adrenalin in contracting crab melanophores is therefore not a puzzling phenomenon. In fact, it is almost surprising that in one case (7) (4), intermedin produces the same response in crab and vertebrate melanophores.

Do Organs Other than the Eye-stalk Produce the Hormone?

This question has been discussed in detail (5) but as yet no conclusive answer to it has been given. It has been reported (9) that ventral nerve cord extracts of *Palæmonetes* are slightly active on the chromatophores of blinded *Palæmonetes*, and therefore that the ventral nerve cord produces the active principle or principles. A criticism of this conclusion is the possibility that the extracts, prepared from normal animals, may have contained traces of E.S.H. present in the blood bathing the nerve cord.

We have extracted several organs of normal pale, normal dark, and blinded crabs (blinded previously for various periods of time), and tested the extracts on crabs blinded previously for from 2–8 days. For purposes of comparing the potency of any organ found to be active with that of the eye-stalk, the minimal unit of activity as already described was employed. The wet weight of an eye-stalk is about 6.0 mg., and consequently 600 cc. ÷ 6 mg. or a 0.001 per cent solution of the eye-stalk represents a minimal unit. Stomach, liver, muscle and heart tissue of both normal dark and blinded specimens were extracted by the usual method and prepared in a 0.1 per cent solution; in other words, 100 times more concentrated than that representing a minimal unit of eye-stalk extract. All extracts were found to be completely inactive. Finally, we resorted to extraction of entire, normal dark crabs, immediately after extirpating the eye-stalks. The extract was prepared in a 2 per cent solution, or 2,000 times more concentrated than the minimal unit of eye-stalk extract. When tested, it was found to be 5 times stronger than the minimal unit. Repetition of this experiment with normal pale animals and animals blinded for from 2 days to 1 month gave identical results. We must conclude that either the hormone is present or being produced by some tissue in the body even in the absence of the eye-stalks, or that the positive result is an artifact brought about by the injection of a hypertonic solution. If the former is true, it can be of little or no significance in the normal chromatic physiology of these animals, as shown subsequently.

Behavior of Isolated Leg Melanophores

The behavior of isolated scale melanophores of fish to various ions and organic substances has been studied (10) for a long time. One of the most remarkable results is the behavior of *Fundulus* scale melanophores to sodium and potassium ions, expanding to the former and contracting to the latter. We have studied the melanophores of crabs to various substances by cutting off the legs with a fine scissors at a point near the articulation of the femur with the body, and immersing these isolated legs into various solutions. The results are summarized in Table II, readings being taken every fifteen minutes after immersion in the solutions and continued for 5 hours.

The results are not particularly illuminating. It is significant, however, that the effect of distilled and tap water is similar to that produced when they are injected into the animal. Sea water also acts in the same fashion as when injected into pale animals. The fact that dark legs paled in sea water after 1½ hours may be due to the diffusion, destruction, or inactivation of E.S.H. already present in the legs. This resembles the last phase of a typical response of blinded crabs to the injection of E.S.H., for when the melanophores begin to contract it requires about 1½ hours to attain complete pallor (5). In view of this, and the slowness of melanophore reaction in crabs, we cannot speak of "ionic effects" on melanophores provided the responses are produced well within an hour. Thus, lithium, sodium, potassium, and calcium chlorides all produce a rapid contraction of expanded melanophores, and consequently, there is no difference, as found in fish melanophores, between the effects of NaCl and KCl. Melanophores in the legs of blinded animals remain more or less contracted in these solutions for 4 hours, so that the effect of these various cations is the same regardless of the original state of the melanophores. However, since the chloride ion is the common anion to all four salts, the result may well be due to it. We have not pursued this aspect further for it is outside the scope of this paper. Isolated leg melanophores of crabs are not valuable material for such an investigation. Their responses are slow, and usually irregular. Most frequently the

TABLE II

Legs of blinded crabs (melanophores contracted)

Solutions		Results of Immersion
$MgCl_2$	.37 M	No change for 4 hours, then expansion of femur melanophores
Sucrose	1.30 M	Same as above, although erythrophores expand within 30 minutes
LiCl	.52 M	No change
Na_2HPO_4	.40 M	Expansion within 15 minutes
NaCl	.52 M	Slight stellation for first hour, then contraction
$CaCl_2$	.34 M	No change for 4 hours, slight expansion on fifth hour
KCl	.53 M	Slight stellation for 1½ hours, then contraction
Sea water		No change during 5 hours
Tap water		Expansion within hour, lasting for 5 hours on femur
Distilled		Same as above

Legs of normal dark crabs (melanophores expanded)

Solutions		Results of Immersion
$MgCl_2$	.37 M	Slight contraction in 15 minutes to 4 hours, then expansion on femur
Sucrose	1.30 M	Slight contraction in ½ hour, expansion after 2 hours
LiCl	.52 M	Contraction in 15 minutes to 5 hours
Na_2HPO_4	.40 M	Remain expanded for 5 hours
NaCl	.52 M	Contraction in 15 minutes to 5 hours
$CaCl_2$	.34 M	Contraction 15 minutes to 4 hours, then expansion on femur
KCl	.53 M	Contraction 15 minutes to 5 hours
Sea water		Contraction after 1½ hours
Tap water		Slight contraction after 1½ hours, but expansion after 4 hours
Distilled		Same as above

reaction starts at the cut surface of the femur and progresses inwardly towards the tibia. Occasionally, the melanophores of the entire femur respond simultaneously, and rarely do the pigment masses of the tibia react prior to those of the femur.

Is the Pale State Constant in Blinded Animals?

It was stated previously that if tissues other than the eye-stalk are capable of producing E.S.H. they must play an insignificant part, if any, in the normal chromatic physiology of the animals. The basis for this belief is that animals once blinded remain in a pale state as long as they remain alive. In all of the experiments, 15–20 blinded animals were maintained in large crystallizing dishes containing about ½ inch of sea water which was changed daily. Under these conditions, blinded animals have been maintained for 2 months, during which they remained entirely pale.

When blinded animals die, they turn quite dark. Even previous to death, the animals turn gradually dark, and remain dark for some time after death. After observing several hundred blinded animals from day to day, we can state that pallor after enucleation of the eye-stalks is largely constant so long as the crabs remain in a healthy state. Thus far, we have not observed regeneration of the eye-stalk, although several puzzling observations, exceptions to the above statement, have suggested this as a possible explanation. The following protocol illustrates the condition of the melanophores in blinded animals:

Aug. 31—158 animals blinded
Sept. 1—100% pale
Sept. 2—98% pale; 2% dark (dead)
Sept. 4—1% slightly dark; 99% pale
Sept. 5—during day 100% pale; during night 100% pale
Sept. 6–13—animals divided into 4 groups:
    white background during day —100% pale
    white background during night—100% pale
    black background during day —100% pale
    black background during night—100% pale
Sept. 13—142 animals remaining:
    3% slightly dark } = A
    5% fully dark }
    92% pale = B
Sept. 14—A = 45% dead; 55% dark; B = 93% pale
Sept. 15—A = 33% dark; 66% pale (day)
    100% pale (night)
Sept. 16—A = 84% dark; 16% pale (day)
    84% pale; 16% dark (night)

The protocol illustrates that over 90 per cent of the animals blinded for 2 weeks remain continuously pale. From 2 per cent–4 per cent, at the end of two weeks, show a slight diurnal rhythm. Further proof of this behavior was obtained by placing 40 animals, blinded two weeks previously, in a glass dish near a window and 25 similar animals in a glass dish in total darkness.

	Exposed to Normal Day and Night Conditions	Darkness
Sept. 14 P.M.	95% P; 5% slightly D	100% P
Sept. 15 A.M.	95% P; 5% slightly D	100% P
Sept. 15 P.M.	95% P; 5% slightly D	100% P
Sept. 16 A.M.	70% P; 30% slightly D	100% P
Sept. 16 P.M.	97% P; 3% slightly D	100% P
Sept. 17 A.M.	67% P; 33% slightly D	100% P

P = pale. D = dark.

A certain percentage of blinded animals is therefore capable of undergoing periodic changes in color. Usually the extent of melanophore expansion is only slight stellation, yet this is a positive reaction. On examination, it was noticed that the stubs of the eye-stalks in those animals showing diurnal rhythm had healed, and were somewhat elongated. However, the stubs were scraped, cut off, or pulled out of their sockets without altering the slightly dark coloration, and consequently the possibility that the regeneration of the sinus gland had taken place was eliminated. That the slight darkening observed in blinded animals during daylight may be due to the direct action of light is improbable because this phenomenon is limited to only a few animals and occurs only after some time has elapsed following extirpation.

Immersion of Animals in Distilled Water

Fifteen blinded animals were placed in a large dish containing distilled water to a depth of one inch, which was changed daily. After 2 days of this treatment, 9 of the animals became dark (ranging from intermediate to complete darkness) while 6 remained pale. These were segregated, and listed as *A* and *B* groups respectively. On the third day, 4 of the animals in *A* were placed in sea water and designated as *C*, and the 3 groups watched daily:

	A (5 animals)	*B* (6 animals)	*C* (4 animals)
4th day	All dark	1 pale, 5 dark	All pale
5th day	4 dark 1 pale	1 pale, 5 dark	All pale
6th day	3 dark 2 pale	2 pale, 4 dark	All pale

At the end of the week, one-third of all the animals had died, the remaining ten were placed in one dish, containing distilled water. On the eighth day, 6 had become pale, and on the ninth day all had become pale, remaining in this state for 4 more days when all but 2 died. One was now injected with distilled water, which promptly evoked an expansion within 15 minutes, but this response vanished within the next 15 minutes. The other was injected with 0.1 E.S. and responded in 15 minutes, remaining dark for 2 hours.

Distilled water has then the same melanophoric effect when animals are immersed in it as when it is injected into the body spaces. After a week, however, the animals become refractory and the dark coloration is gradually lost, but the animals have not lost the ability to respond to injections of E.S.H. or distilled water.

Chemical Characteristics of E.S.H.

The properties of E.S.H. reported earlier (5) have been confirmed and extended. However, in our experiments, the solubility of E.S.H. in various solvents was determined after the following preliminary treatment: 100 eye-stalks were extracted three times with 10 cc. of distilled water by boiling. The solution was filtered and the filtrate reduced to a volume of 10 cc. under a warm current of air. Two volumes of either pyridine, ethanol or acetone were added, and the solution cooled to 5° C. for one day. A heavy red precipitate forms which is discarded after centrifugation. The supernatant fluid contains practically all of the activity, and after drying, was used as stock material. One extraction with 10 cc. of the following solvents was made of the dry powder equivalent to 10 E.S., and both soluble and insoluble fractions assayed:

Solvent	Percentage of Activity Soluble
	per cent
Absolute ethyl alcohol	45
Absolute methyl alcohol	60
95% ethyl alcohol	60
Absolute acetone	0
Ethyl ether	0
Petroleum ether	1
Benzene	1
Chloroform	0
Ethyl acetate	0
Pyridine	6
95% acetone	2
90% acetone	20
95% methyl alcohol	80

Due to the small amount of material available, we have not concentrated on the purification of the hormone. However, if the stock material is repeatedly extracted with 95 per cent methyl alcohol, most of the activity can be collected as a soluble fraction, which, on basis of activity per milligram of dry weight, represents a tenfold purification.[3] Aqueous acetone seems to destroy some of the hormone for we have not been able to account for all of the original activity present before fractionation by totalling the activity of the soluble and insoluble fractions.

The hormone is apparently soluble in water throughout the pH range. Stock material (20 E.S.) was easily dissolved in 10 cc. of 0.1 N NaOH as well as in 10 cc. of 0.1 N HCl with the formation of a flocculent, inactive precipitate. If 10 E.S. are dissolved in 10 cc. of 0.1

TABLE III

Diurnal rhythm of *Uca pugilator*

Time of Observation	Normal Day and Night Conditions				Constant Darkness	
	Normal Animals			Animals Blinded 2 Weeks Previously *	Normal Animals	Blinded 2 Weeks Previously
					Indeterminate Background	
	White Background 40 Animals	Black Background 40 Animals	Indeterminate Background 40 Animals	Indeterminate Background 40 Animals	25 Animals	25 Animals
September 13 11:00 A.M.	100% D	100% D	100% D	75% I-P	100% D	4% D
7:30 P.M.	82% P	100% D	100% D	100% P	—	—
12:45 A.M.	100% P	50% I; 50% P	80% P	100% P	50% P; 50% I	100% P
September 14 10:00 A.M.	100% D	100% D	100% P	70% I-P	100% D	100% P
8:00 P.M.	26% P; 74% I	12% P; 88% P-I	80% P	98% P	60% P	100% P
10:00 P.M.	80% P; 20% I	18% P; 82% I-D	100% P	98% P	80% P	100% P
September 15 10:00 A.M.	100% D	100% D	100% D	50% I-D	100% D	100% P
4:30 P.M.	100% D	98% D	100% D	87% P	25% P	100% P
10:30 P.M.	90% P	50% P	66% P	87% P	92% P	100% P
September 16 11:45 A.M.	100% D	100% D	100% D	40% I	96% D	100% P
10:30 P.M.	100% P	25% P	100% P	90% P	84% P	100% P
September 17 10:30 A.M.	100% D	100% D	100% D	40% I	84% D	100% P

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[3] It was reported (8) that the hormone was precipitated from 95 per cent acetone by the addition of ether. This is an error since in this case the hormone was precipitated from 95 per cent methanol, not 95 per cent acetone.

TABLE III—*Continued*

Time of Observation	All Animals Now Exposed to Constant Light (75 Watt Electric Bulb)					
	Normal Animals			Animals Blinded 2 Weeks Previously *	Normal Animals	Blinded 2 Weeks Previously
					Indeterminate Background	
	White Background 40 Animals	Black Background 40 Animals	Indeterminate Background 40 Animals	Indeterminate Background 40 Animals	25 Animals	25 Animals
September 18 11:15 A.M.	100% D	100% D	100% D	40% I	100% D	55% I-P
3:00 P.M.	98% D	100% D	100% D	25% I	100% D	50% I-P
10:15 P.M.	40% P; 60% I-D	14% P	33% P	3% I; 97% P	33% P	100% P
September 19 12:30 P.M.	100% D	100% D	100% D	25% I	100% D	25% I-P
10:00 P.M.	30% P	20% P	50% P	92% P	50% P	100% P
September 20 12:30 P.M.	100% D	100% D	100% D	12% I	100% D	25% I-P
11:00 P.M.	75% P	33% P	75% P	92% P	50% P	75% P
September 21 12:30 P.M.	100% D	100% D	100% D	14% I	100% D	50% I
10:30 P.M.	50% P	20% P	50% P	90% P	33% P	90% P
September 22 12:40 P.M.	100% D	100% D	100% D	16% I	100% D	42% I
10:20 P.M.	50% P	16% P	60% P	94% P	50% P	90% P
September 23 11:10 P.M.	50% P	6% P	33% P	91% P	33% P	100% P
September 25 1:00 A.M.	50% P	14% P	80% P	96% P	12% P	75% P

P = pale.
D = dark.
I = intermediate.
I–D = intermediate to dark.
I–P = intermediate to pale.

* These were chosen from several hundred specimens because they were not entirely pale.

NaOH and, after 3 minutes, neutralized to pH 7.0 and tested, complete activity is found. If a similar solution is placed at 100° C. for ten minutes, total loss of activity occurs. Ten E.S. in 10 cc. of 0.1 N HCl placed at 100° C. for ½ hour, neutralized, and tested, retains its original activity. In fact, HCl seems to potentiate the hormone somewhat, although we have not studied this carefully. Attempts to regenerate the activity of stock material treated for 15 minutes with NaOH by boiling it with an equivalent amount of HCl after neutralization were unsuccessful.

The Diurnal Rhythm of Uca pugilator

It has been stated (5) that the diurnal chromatic rhythm of *Uca pugilator* proceeded under constant illumination or darkness, and regardless of background. This statement was based upon observations made on only the melanophores of the abdominal segments, since the ventral surface of these crabs can be most easily observed. Since all the experiments reported here deal with the reactions of leg melanophores, we studied for 2 weeks the behavior of the leg melanophores during the periodic change in coloration. Essentially, the same situation as previously described was found, but several new facts of interest were discovered. The following protocol summarizes the observations upon 170 specimens kept under various conditions:

It will be seen that when normal animals are exposed to normal day and night environment, the diurnal rhythm proceeds as described previously on both white and indeterminate backgrounds. On black background, however, the appearance of the nocturnal hue is delayed and does not occur in a certain percentage of animals. Under constant light, the same situation is true except that fewer animals on all backgrounds show the nocturnal phase. Animals maintained in darkness show the nocturnal coloration at night but when exposed to constant light, the number of specimens becoming pale at night is decreased. Two new facts therefore emerge: (1) that black background delays and inhibits the pale phase more effectively under conditions of constant illumination than under normal condition daylight and darkness, (2) that constant light does the same regardless of background.

Observations on the ventral surface of these specimens were made simultaneously but excluded from the protocol. If readings were based upon the behavior of the abdominal melanophores alone, the protocol would indicate that the pale phase occurred in practically all of the specimens regardless of background or of conditions of illumination. There is, therefore, a decided difference between the reactions of the leg and abdominal melanophores.

The diurnal rhythm of *Uca* has been explained on the assumptions that during the day, E.S.H. is released into the blood stream and that during night, release is stopped. On this basis, it must be assumed that the abdominal melanophores are more sensitive than those of the legs to the disappearance of the hormone from the blood stream. In addition to the circumstantial evidence obtained by watching single animals or groups of animals during the diurnal rhythm, further proof was obtained by removing the eye-stalks of 15 dark animals during the day. After 1 hour, 20 per cent of the animals showed white abdomens but the legs remained dark in all cases. After 1½ hours, 66 per cent of the specimens showed white abdomens and 20 per cent, pale legs. After 2½ hours, all showed pale abdomens while 70 per cent showed pale legs. This experiment, therefore, indicates quite clearly that the abdominal melanophores respond more rapidly than leg melanophores to the loss of E.S.H. from the blood, as brought about by removal of the eye-stalks.

The assumption that the pale phase of *Uca* is due to the absence of E.S.H. from the circulation during the night is based on no direct evidence inasmuch as we have not succeeded in obtaining the blood of *Uca* for assaying E.S.H. The evidence for this belief is, therefore, indirect, and is based on one fact—that pallor in *Uca* is always associated with and due to the loss of the eye-stalks, and hence removal of the chief if not the only significant source of E.S.H. However, it may be possible that the melanophores become refractory during the night to the eye-stalk hormone (which may be thought of as being secreted constantly) thus ushering in the pale phase, or that the pale phase is due to a melanophore-contracting hormone, concentrating nerves, or some unknown factor. The first possibility was negated by injecting some E.S.H. into 20 normal animals showing the nocturnal phase. All responded normally by darkening. The second possibility has no experimental basis inasmuch as no extract of *Uca* has been prepared which induces pallor, and as chromatophore nerves have not been demonstrated in the Crustacea.

Consequently, the explanation of the diurnal rhythm of *Uca* on the basis of the presence and absence in the blood stream of one hormone (E.S.H.) seems to be the most economical, and in conflict with no observational and experimental facts. The effect of constant light and of black background can therefore be thought of as prolonging the release of the eye-stalk hormone into the circulation, or as inhibiting or delaying the process by which the release of the hormone from the gland is stopped.

DISCUSSION

Since most of the data have already been analyzed, we would limit this to a short theoretical discussion on the nature of action of the eye-stalk hormone. It is evident that the problem of the endocrine control of chromatophores in crustaceans is not as simple as has been regarded. While in general the comparative endocrinology of melanophore responses in both vertebrates and invertebrates is rather well understood, we have no inkling of the microphysiology of either the melanophores or the chromatophorotropic hormones in action. In fact, there is nothing known, with one or two exceptions, concerning the intimate mechanism by which a hormone is able to affect a tissue. The finding that distilled water expands the melanophores of blinded crabs may furnish a clue as to the manner in which the eye-stalk hormone acts, even though the reactions may not be comparable. Since the characteristic action of E.S.H. is the induction of protoplasmic movements in the melanophores, one is inclined to suspect the ionic or osmotic environment as the regulator of melanophore movements. This may or may not be reasonable but, at least, the effect of distilled water can be attributed to the resulting hypotonicity of the blood which in turn might cause a rapid shift in the osmotic equilibrium between the blood and tissues. The expansion of the melanophores might then result from such a change in the osmotic or ionic environment.

There are two possibilities concerning the way in which E.S.H. acts on the melanophores—directly or indirectly. Discussing the second possibility first, it is quite possible that the eye-stalk hormone may not have any relation to changeable coloration at all, but that it regulates the salt or water balance of the animal. The effect on melanophores may well be indirect, resulting from osmotic or ionic changes between the blood and tissues when the hormone is normally present and absent in the blood stream. This would mean, of course, that the chromatophorotropic hormone is only secondarily chromatophorotropic; its main function being in regulating some other process. Indeed, it has been maintained (11) that since this hormone is found in crustaceans which do not show color changes or even contain chromatophores, it must perform some function more important than that of regulating color changes. Such an argument is helpful for the above discussion, but we feel that the presence of a particular hormone may not necessarily mean that it must be performing some function. For example, intermedin is abundantly present in the pituitary glands of both birds and mammals, yet its presence is not correlated with any functional significance. Similarly, the presence of estrogenic hormones in the mammalian testes does not seem to be correlated with any of the normal processes in the reproductive physiology of the male. There are other instances of this kind, but of course some function for the hormones mentioned may be discovered eventually.

To the effect that E.S.H. acts directly on the melanophores, we might add that all of our curves relating response to concentration and time are of a type which has been assumed to represent reversible compound formation on cell surfaces—but this is all that we can marshal in support of a direct action. Isolated leg melanophores will expand when immersed into a solution of sea water containing E.S.H., but this is of course no indication of a direct action. Similarly, the effectiveness of various salts on isolated melanophores offers nothing to indicate that the action of E.S.H. is direct or indirect. The determination of the mechanism of action of the eye-stalk hormone belongs properly to general physiology and is perhaps one of the most significant aspects of melanophore physiology. It is hoped that some of these observations will furnish a basis for further investigation.

In concluding, it can be mentioned that a search for other endocrine functions of the eye-stalk such as have already been found (regulation of heart (12), calcium metabolism (13)) will be of importance in establishing the proper rôle which the eye-stalks play in regulating the pigmentary effectors. The hormone is not indispensible to the life of crustaceans. Blinded crabs molt frequently (perhaps more frequently than normal specimens according to our impression) and if well fed can be maintained in a healthy state for several months, and perhaps indefinitely.

Summary

1. A method for determining within an hour the relative activity of eye-stalk extracts of *Uca pugilator* has been described.

2. The amount of hormone present in one eye-stalk of *Uca* (5.0 grams) was calculated to be 0.2 γ. The minimal amount of hormone detected by the method described was calculated to be 0.000016 γ, which represents a minimal unit of eye-stalk activity.

3. Distilled water is effective in expanding melanophores when injected into blinded specimens. The effect seems to be related to osmotic changes induced in the animal. Excepting the effect of distilled and tap water, the response of blinded animals to the eye-stalk hormone seems to be quite specific. Sixteen different drugs were ineffective in causing melanophore expansion.

4. Blinded animals immersed in distilled water become and remain dark for a week, after which they become refractory. However, they have not lost the ability to respond to injections of either eye-stalk hormone or distilled water.

5. Extracts of several organs other than the eye-stalk were found to be inactive on blinded specimens, even when 100 times more concentrated than a minimal unit of eye-stalk extract. Extracts of entire bodies, 2,000 times more concentrated than a minimal unit, were active.

6. Isolated leg melanophores respond slowly and irregularly to various ions. Na, K, Ca, Li chlorides all induce melanophore contraction. Na and K do not produce opposed responses in isolated leg melanophores as they do in isolated scale melanophores.

7. The relative solubility of the eye-stalk hormone in various organic solvents was determined. The hormone is inactivated by alkali, but the activity cannot be regenerated by treatment with acid.

8. Animals blinded for 2 weeks or more remain continuously pale, with the exception of 2–4 per cent of the specimens, which show slight periodic changes in coloration.

9. The diurnal rhythm of *Uca* is affected by constant light, and black background, both of which delay the appearance of the nocturnal hue on the legs.

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THE EFFECT OF SODIUM, POTASSIUM AND CALCIUM IONS ON CHANGES IN VOLUME OF AMOEBA PROTEUS[1]

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INTRODUCTION

McCutcheon and Lucké (1928) found that, with the same osmotic concentration, permeability of the eggs of *Arbacia* to water is greater in solutions of dextrose containing 0.01 M NaCl or KCl and less in solutions of dextrose containing 0.004 M $CaCl_2$ or $MgCl_2$ than in solutions of dextrose in distilled water. These results indicate that sodium and potassium ions increase and calcium and magnesium ions decrease permeability to water. Jacobs (1930) showed that hemolysis of erythrocytes in solutions of non-electrolytes, 0.01 M or 0.02 M, is almost as rapid as it is in distilled water; but that in solutions of electrolytes, e.g. NaCl 0.001 M or $CaCl_2$ 0.0001 M, it is considerably slower. These results indicate that sodium as well as calcium ions decrease permeability to water, i.e. that under some conditions monovalent as well as divalent salts cause decrease in permeability.

The experiments considered in the following pages were undertaken to ascertain the effect of sodium, potassium and calcium ions in phosphate buffer solutions (0.002 M, pH 6.8) on changes in the volume of *Amoeba proteus*.

MATERIALS

Amoeba proteus was used exclusively in these experiments. It was grown in cultures prepared according to the method described in an earlier paper (Mast and Fowler, 1935). The distilled water used was triple redistilled from a tandem glass still (Mast, 1928). The solutions used were prepared by making two stocks, one containing the primary phosphate of the cation to be tested, 0.002 M, and the other containing the hydroxide of the same cation, 0.002 M, and mixing the two stocks

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[1] This paper is the second in a series on the relation between environmental factors and water-content in *Amoeba proteus*. The first paper in the series, entitled "Permeability of Amoeba proteus to Water," was published in the *Journal of Cellular and Comparative Physiology*, Vol. 6, pp. 151–167, 1935.

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in the proportions necessary to maintain the hydrogen ion concentration at pH 6.8. The preparation and standardization of the hydroxide stock solutions is described in the paper cited above. All the solutions thus prepared contained the same concentration of the metallic cation used and the osmotic concentration of these solutions was practically the same as that in which the amoebae were grown, i.e. modified Ringer solution.

If the salts in the solutions used had been completely ionized, the osmotic concentration of the solutions containing calcium would have been greater than those containing either sodium or potassium because if $CaH_4(PO_4)_2$ ionizes there are produced two phosphate ions for each calcium ion, whereas if KH_2PO_4 or NaH_2PO_4 ionizes there is produced only one phosphate ion for each potassium or sodium ion. At the concentration used, however, the sodium and the potassium compounds were slightly more ionized than the calcium compounds. The number of ions per unit volume and the osmotic pressure were probably therefore practically the same in the solutions used.

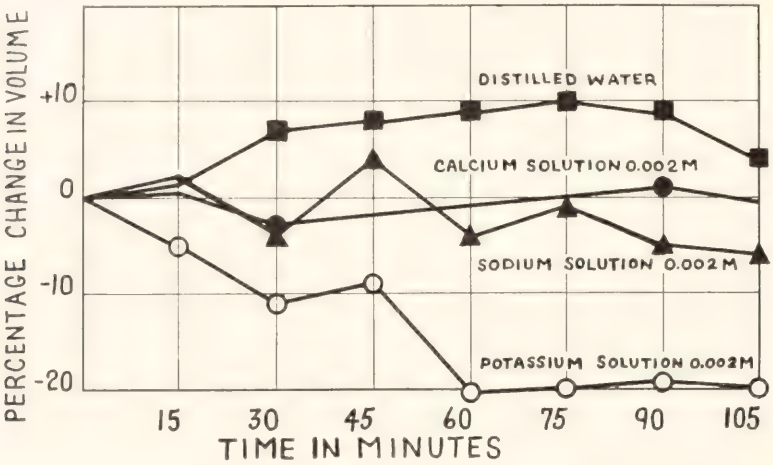

FIG. 1. Graphs showing the relation between sodium, potassium and calcium respectively in phosphate buffer solutions (pH 6.8) and changes in volume in *Amoeba proteus* transferred from dilute Ringer solution of the same osmotic concentration. Each point on the curves represents the average of the results of three measurements made on three different individuals. Ordinates, change in volume as percentage of the original volume; abscissæ, time in minutes after transfer from dilute Ringer solution.

METHODS AND RESULTS

Three methods were used in ascertaining the effect of these solutions on changes in volume of *Amoeba*.

1. Six amoebae were prepared, selected, and measured individually in modified Ringer solution as described in the paper cited above (Mast and Fowler, 1935). Then they were transferred to distilled water and measured at fifteen-minute intervals. Then this was repeated with other individuals, using in place of distilled water phosphate buffer solutions which contained sodium, potassium and calcium respectively, but with only three individuals in each. The results obtained are presented in Fig. 1.

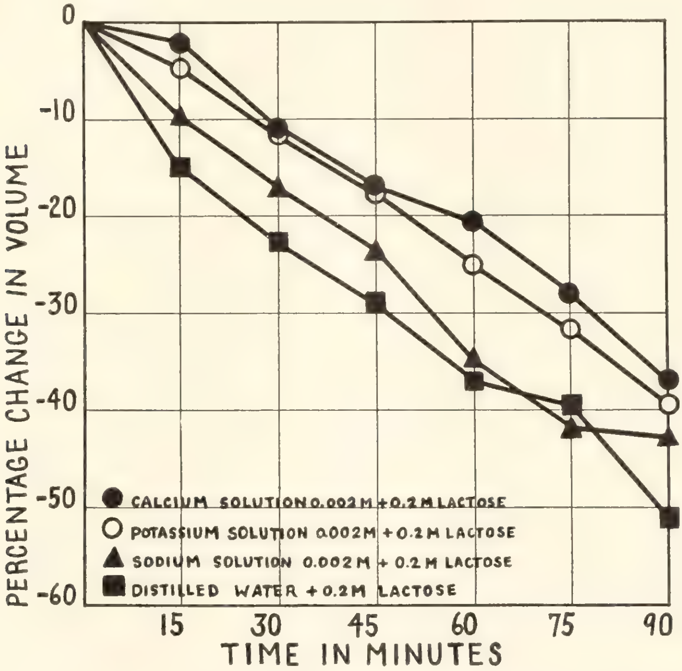

FIG. 2. Graphs showing the relation between sodium, potassium and calcium respectively in hypertonic solutions of lactose (pH 6.8) and changes in volume in *Amoeba proteus* transferred from dilute Ringer solution.

Each point on the curves represents the average of the results of ten measurements made on 7 to 12 different individuals. Ordinates, change in volume as percentage of the original volume; abscissæ, time in minutes after transfer.

Figure 1 indicates that the amoebae transferred from modified Ringer solution to distilled water increased 10 per cent in volume in 75 minutes; that those transferred to the calcium phosphate buffer solutions of practically the same osmotic concentration as the Ringer solution, did not change appreciably in volume during the time represented in the graph, and that those transferred to the sodium phosphate buffer solution decreased only slightly if at all during this time, but that those transferred to the potassium phosphate buffer solution of the same osmotic concentration as the preceding solutions, decreased 20 per cent in 60 minutes and then remained constant.

After the experiment had continued 105 minutes, the amoebae in the distilled water and in the calcium phosphate buffer solution were normal and they continued to live for several days; those in the sodium phosphate buffer solution soon began to round up and decrease in volume and they disintegrated about 60 minutes later; and those in the potassium buffer solutions disintegrated even sooner. However, those in all the solutions recovered fully in culture solution, if they had not been in the salt solutions more than one hour. The results obtained show, therefore, that without any change in osmotic concentration potassium causes rapid loss of water in *Amoeba*, without any appreciable injury; and that sodium and calcium have little if any effect.

TABLE I

Percentage changes in volume in amoebae transferred from modified Ringer solution (0.002 M, pH 6.8) to a solution containing sodium as the only metallic cation, of the same hydrogen ion and osmotic concentration as the Ringer solution, plus 0.2 M lactose. Temperature, 22.7°–25.5°; E_m, probable error.

Designation of specimen	Time in minutes after transfer						
	15	30	45	60	75	90	105
1	− 7	−17	−17	−27	−32	−32	−46
2	− 2	−18	−37	−48	−55	−60	−55
3	−13	−20	−25	−29	−34	−46	−46
4	−14	−23	−26	−40	−53	−52	−62
5	− 8	−24	−45	−61	−63		
6	−17	−20	−28	−26	−33	−35	−43
7	+ 1	− 9	−21	−31	−36	−41	−44
8	−16	− 9	− 7	−41	−36	−27	−27
9	−18	−26	−23	−34	−41	−39	−51
10	− 3	− 5	− 8	−16	−41	−58	
Mean	−10	−17	−23	−35	−42	−43	−47
E_m	±1.382	±1.442	±2.36	±2.58	±2.18	±2.44	±2.28

2. Several amoebae were prepared, selected, and measured as in method 1, and then transferred to 0.2 M lactose in distilled water, and measured at fifteen-minute intervals. Then this was repeated with phosphate buffer solutions which contained lactose (0.2 M) and sodium, potassium and calcium (0.002 M) respectively. The results obtained are presented in Fig. 2 and Tables I, II, and III.

Figure 2 indicates that the amoebae which were transferred from modified Ringer solution to hypertonic solutions, consisting of lactose in distilled water and respectively sodium, potassium and calcium plus lactose, decreased in volume in all the solutions, but that the decrease was least in the solution containing calcium and greatest in distilled water plus lactose.

Tables I, II, and III show that there was marked individual variation in the reduction in the volume of the amoebae in all the hypertonic solutions tested, but that in spite of this variation, the probable error is relatively so small that there is overlapping at only a few points on the curves. This practically proves that the differences in the means of the reduction in volume in the different solutions, indicated in the curves in Fig. 2, are significant and consequently that the order of effectiveness on preventing loss of water in *Amoeba*, is Ca>K>Na, with very little difference between calcium and potassium.

TABLE II

Percentage changes in volume in amoebae transferred from modified Ringer solutions (0.002 M, pH 6.8) to a solution containing potassium as the only metallic cation, of the same hydrogen ion and osmotic concentration as the Ringer solution, plus 0.2 M lactose. Temperature, 22.1°–25.2°; E_m, probable error.

Designation of specimen	Time in minutes after transfer						
	15	30	45	60	75	90	105
1	−2	−8	−19	−23	−28	−23	−36
2	−10	−21	−24	−30	−28	−35	−33
3	+10	−11	−16	−23	−35	−43	
4	−2	−10	−15	−15	−28	−20	−34
5	−21	−14	−22	−31	−24	−43	−43
6	−11	−17	−23	−32	−42	−57	
7	−11	−14	−11	−30	−37	−43	
8	+10	−4					
9	−4	−4	−11	−29	−37		
10	−17	−17	−17	−20	−20	−46	
11	−9	−9	−16	−20	−34	−43	
12	−11	−11	−18	−24	−32	−42	−58
Mean	−6	−12	−18	−25	−32	−40	−41
E_m	±1.79	±0.97	±0.85	±1.07	±1.26	±2.10	±2.80

The facts that the osmotic concentrations of the solutions which contained respectively sodium, potassium and calcium plus lactose were practically equal and that the osmotic concentration of these solutions was somewhat higher than that of the distilled water plus lactose, show that the monovalent sodium and potassium ions as well as the divalent calcium ions, retard the loss of water, but that calcium ions are more effective than sodium or potassium ions.

3. Ten amoebae were prepared, selected, and measured in modified Ringer solution as in methods 1 and 2, then they were transferred to this solution containing lactose (0.2 M), left 30 minutes and measured again. They were then transferred to distilled water and measured at 30-minute intervals. This whole process was then repeated with other specimens in respectively sodium, potassium and calcium phosphate buffer solutions (0.002 M) in place of distilled water. The results obtained are presented in Fig. 3.

Figure 3 shows that the amoebae which were transferred from modified Ringer solution to modified Ringer solution containing lactose (0.2 M) decreased in volume to about 70 per cent of the original volume in 30 minutes and that after they had been transferred from this solution to distilled water or to phosphate buffer solutions containing sodium, potassium or calcium, they increased in volume, but that the increase was much more rapid and much greater in distilled water than in any of the salt solutions. The figure shows that in all the salt solutions the amoebae increased to a maximum and then decreased, but that increase was greater in the solution which contained sodium than those which contained potassium or calcium, the maximum increase being 22 per cent for sodium and only 9 per cent for potassium and 4 per cent for calcium. It also shows that in distilled water the amoebae nearly regained their original volume in 30 minutes, and then continued to increase as long as measurements were made, i.e. for 90 minutes, and that at the close of the experiment they were 17 per cent larger than at the beginning.

TABLE III

Percentage changes in volume in amoebae transferred from modified Ringer solution (0.002 M, pH 6.8) to a solution containing calcium as the only metallic cation, of the same hydrogen ion and osmotic concentration as the Ringer solution, plus 0.2 M lactose. Temperature, 22.5°–24.0°; E_m, probable error.

Designation of specimen	Time in minutes after transfer						
	15	30	45	60	75	90	105
1	−8	−2	−11	−20	−23	−29	
2	−12	−22	−25	−17	−29	−44	−53
3	−12	−21	−23	−26	−41		
4	+7	−8	−6	−13	−30	−36	−43
5	+4	−5	−8				
6	+7	−18	−27	−29	−32	−38	−47
7	−3	−5	−21	−17	−13	−42	
Mean	−2	−12	−17	−21	−28	−38	−48
E_m	±2.01	±1.98	±2.01	±1.53	±1.93	±1.55	±1.61

The individual variations in the increase in volume in the hypotonic solutions were essentially the same as those recorded in Tables I, II and III for hypertonic solutions, but the differences in the means were greater so that there was no overlapping of the probable errors, except at the last points on the curves. The differences in the means of the increase in volume in the different solutions indicated in the curves in Fig. 3, are therefore significant, showing that the order of effectiveness on gain of water in *Amoeba* in hypotonic solutions is, Ca>K>Na, that is, that it is the same as it is on loss of water in hypertonic solutions.

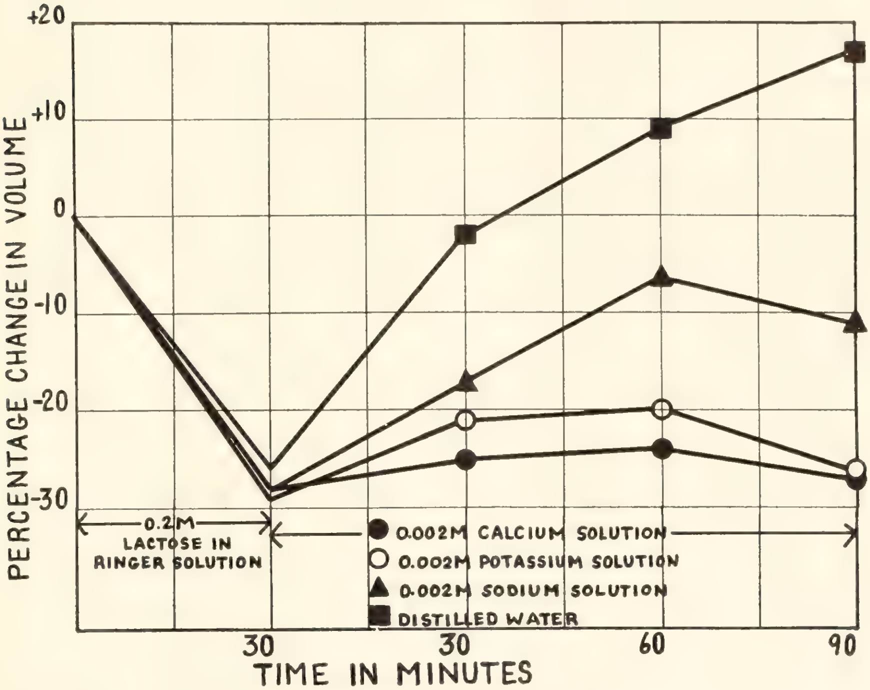

FIG. 3. Graphs showing the relation between sodium, potassium and calcium respectively in hypotonic buffer solutions (pH 6.8) and changes in volume in *Amoeba proteus* transferred from weak Ringer solution containing 0.2 M lactose.

Each point on the curves represents the average of the results obtained in ten measurements made on ten different individuals. Ordinates, change in volume as percentage of the original volume; abscissæ, time in minutes after transfer.

By comparing Fig. 3 with Fig. 1, it will be seen that if *Amoeba* is transferred from Ringer solution (0.002 M) to potassium phosphate solution (0.002 M) it decreases markedly in volume, but that if it is transferred from lactose solution (0.2 M) to potassium phosphate solution (0.002 M) it increases somewhat. This difference is doubtless correlated with the fact that the water-content was much higher in the amoebae which were transferred from the modified Ringer solution than it was in those which were transferred from the lactose solution.

The results presented in Fig. 3 show therefore that monovalent as well as divalent cations retard the entrance of water but that, just as in reference to loss of water, the latter are more effective than the former; and they support the conclusion reached above that both monovalent and divalent metallic cations tend to maintain the water-content in *Amoeba* constant.

DISCUSSION

The question now arises as to how the metallic ions act in their tendency to maintain the water-content of *Amoeba* constant.

Under normal conditions much water is eliminated by the contractile vacuole and considerable water enters with the food in the process of feeding (Mast and Hahnert, 1935) and some possibly enters in the formation of cups without food (Mast and Doyle, 1934). It may be then that the metallic ions influence the water-content of *Amoeba* through action on some or all of these processes. Such action could, however, not have been seriously involved in the results presented in the preceding pages; for in the solutions used there was, during the time the observations were made, no feeding and no formation of cups of any sort and the contractile vacuole did not function except in a few of the more dilute solutions and then only after the amoebae had been in the solution half an hour or more. The changes in water-content observed must therefore have been due to passage of water directly through the surface layer of the amoebae, and the effects of the metallic ions on the rate of the passage through this layer must have been due either to their action on the permeability of this layer or to their action on the osmotic concentration of substances in the amoebae, or to their action on imbibition, but it is probably largely due to the first. This matter will be discussed more fully in a subsequent paper.

The conclusion that monovalent as well as divalent metallic cations tend to maintain the water-content of *Amoeba* constant, is in agreement with the results obtained by Jacobs, who showed that in erythrocytes permeability to water is decreased by the addition of either NaCl or $CaCl_2$ to distilled water; but they are not in agreement with those obtained by McCutcheon and Lucké, who showed that permeability of the eggs of *Arbacia* to water is increased if NaCl or KCl is added to a solution of dextrose in distilled water and decreased if $CaCl_2$ or $MgCl_2$ is added.

Summary

1. If amoebae are transferred from modified Ringer solution (0.002 M, pH 6.8) to distilled water or to phosphate buffer solution containing respectively sodium, potassium and calcium as the only metallic cation (0.002 M, pH 6.8), they increase in size in distilled water, decrease in size in the solution which contains potassium and remain nearly the same in size in the solution which contains sodium or calcium.

2. If they are transferred from modified Ringer solution (0.002 M, pH 6.8) to distilled water containing lactose (0.2 M) or to phosphate buffer solutions containing lactose (0.2 M) and respectively sodium, potassium and calcium as the only metallic cation, they decrease in volume in all the solutions but they decrease most rapidly in distilled water containing lactose, less rapidly in the solution containing sodium and lactose, still less rapidly in the solution containing potassium and lactose, and least rapidly in the solution containing calcium and lactose.

3. If they are transferred from modified Ringer solution (0.002 M, pH 6.8) to Ringer solution containing lactose (0.2 M) and left 30 minutes, they decrease greatly in volume. If they are then transferred respectively to distilled water and to phosphate buffer solutions, containing respectively sodium, potassium and calcium (0.002 M, pH 6.8), they increase in volume in all the solutions, but they increase most rapidly in distilled water, less rapidly in the solution containing sodium, still less rapidly in the solution containing potassium and least rapidly in the solution containing calcium.

4. These results show that under some conditions monovalent as well as divalent cations cause decrease in the rate at which water passed into and out of *Amoeba proteus*. This is probably largely due to their action on permeability to water.

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THE CONTRACTILE VACUOLE IN AMOEBA PROTEUS (LEIDY)

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Metcalf (1910) maintains that there are scattered through the cytoplasm of *Amoeba proteus* numerous small round granules called microsomes or cytomicrosomes, and that the "contractile vacuole is surrounded by a layer of granules of the same size and appearance as the microsomes of the general cytoplasm." He says (p. 302): "When the vacuole is of moderate size these granules form a continuous layer, one granule thick," when it is large there are open spaces between the granules, and when it is small the layer is several granules thick. He maintains that during contraction these granules form a clump in which the new vacuole develops and consequently concludes that they "are connected functionally with excretion," and he therefore calls them "excretion granules." He admits, however, that the contractile vacuole can arise and function without the granules. He says (p. 307); "The results of operations upon Amoeba show that a new vacuole in an Amoeba fragment appears first and that only gradually the granules collect about it. The granules are not essential to the functional vacuole."

Mast (1926) confirmed Metcalf's contentions concerning the form, size and distribution of the granules in question, but he holds that the conclusion that they function in excretion is not well founded and that their aggregation on the surface of the contractile vacuole is a purely physical phenomenon. He designated them beta granules to differentiate them from somewhat similar but much smaller granules which he designated alpha granules.

Mast and Doyle (1935*a*, *b*) demonstrated that the beta granules have staining properties like the granules in other cells, known as mitochondria. They centrifuged amoebae and found that all the beta granules moved through the cytoplasm from the axis of rotation and that those on the contractile vacuole moved to the centrifugal surface and formed a considerable mass there, and they obtained some evidence indicating that the beta granules on the surface of the contractile vacuole are imbedded in a layer of substance which is somewhat more viscous than the adjoining substance in the cytoplasm and moves with them during the process of centrifuging. They conclude that the beta granules function in transferring substances from place to place in the cytoplasm and that they probably thus facilitate excretion of substance by the contractile vacuole.

METHODS

I made at various times numerous detailed observations on the contractile vacuole in *Amoeba proteus* under the best optical system obtainable (oil immersion apochromatic objective, 60 × and 90 ×, and compensating oculars, 10 ×, 15 × and 20 ×). All the observations were made on living specimens. Some of the specimens had been without food for several days and contained no food vacuoles and but little fat and relatively few granules and crystals. Some of these were greatly flattened by gradually removing water from under the coverglass. Under these conditions the contractile vacuole and the granules and other substances around it could be very clearly seen. The following results were obtained.

RESULTS

The contractile vacuole is usually surrounded by a layer of beta granules (Metcalf's excretory granules). These granules are imbedded in a layer of substance which is optically distinctly different from the adjoining cytoplasm. It has a slightly yellowish tint and appears to consist of coagulated substance. It is more viscous and heavier than the rest of the cytoplasm for it moves to the centrifugal surface of the contractile vacuole when amoebae are centrifuged (Mast and Doyle, 1935*b*, Fig. 1). When the contractile vacuole is fully developed the granules around it usually form a layer one granule thick, with some spaces between the granules here and there. As the vacuole contracts these spaces disappear, the layer of granules becomes continuous and the layer of substance in which they are imbedded thickens. As it contracts further this layer becomes still thicker and the granules pile up on each other until there is, when contraction is complete, a distinct mass of substance of considerable size, in which a remnant of the vacuole can usually still be seen. That is, the vacuole usually does not completely disappear during systole and soon begins to enlarge again. If it disappears completely a new vacuole usually appears and develops in the mass of substance in which the old one disappeared (Fig. 1). This, with the exception of the layer of substance in which the granules are imbedded, is in full accord with Metcalf's views (1910).

At the surface of the contractile vacuole under the layer of substance containing the beta granules, there is a layer or membrane about

0.5μ thick which is optically well differentiated from the adjoining substance on either surface, for under favorable conditions a line indicating an interface can be clearly seen at both of these surfaces. The beta granules are never in close contact with this membrane.

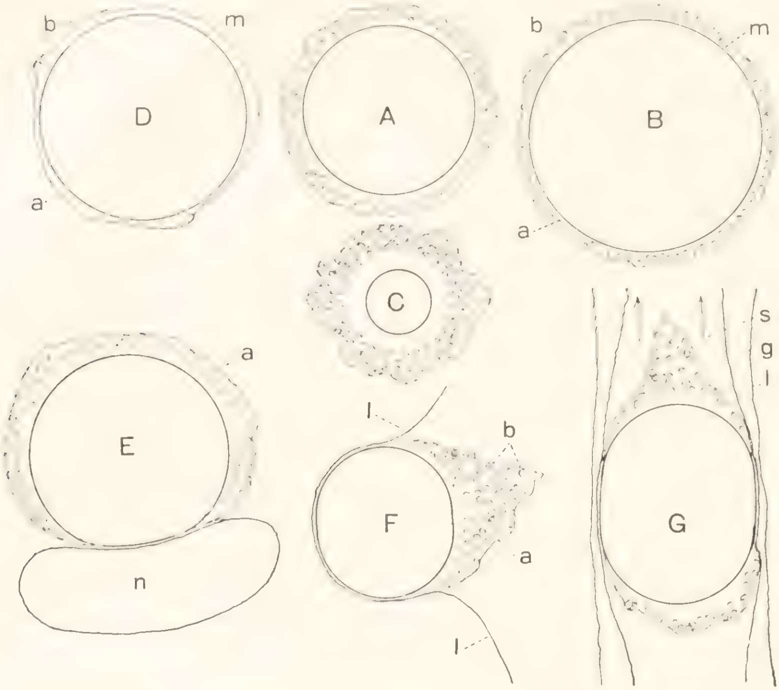

FIG. 1. Camera drawings of the contractile vacuole in *Amoeba proteus* showing the relation between the vacuole and the surrounding cytoplasm, under different conditions and in different phases. *m*, membrane on the surface of the vacuole; *a*, layer of differentiated cytoplasm around the vacuole; *b*, beta granules (mitochondria) imbedded in this layer; *l*, plasmalemma; *g*, plasmagel; *s*, plasmasol; *n*, nucleus.

A, contractile vacuole partially expanded; *B*, same vacuole fully expanded; *C*, same vacuole shortly after contraction; *D*, a vacuole which had only a few beta granules in the differentiated layer at the surface; *E, F, G*, contractile vacuoles which are in contact with other structures.

Note that the beta granules are not in contact with the membrane at the surface of the vacuoles, that they differ greatly in number in different vacuoles, and that the layer of differentiated cytoplasm in which they are imbedded becomes much thicker as the vacuole contracts. Note also that when the contractile vacuole comes in contact with other structures the differentiated layer around the vacuole is pushed aside.

They are always separated from it by substance in the layer in which they are imbedded. This separation is least when the contractile vacuole is fully developed and increases as it contracts (Fig. 1).

If the contractile vacuole comes in contact with an object, the layer in which the beta granules are imbedded is pushed aside and the membrane around it comes in direct contact with the rest of the cytoplasm (Fig. 1). This shows that this layer is only loosely connected with the membrane.

The number of beta granules varies greatly and probably also the amount of substance in which they are imbedded. I have seen a considerable number of fully developed vacuoles which functioned normally in all respects, with only 10 or 12 granules at the surface (Fig. 1), and one in which there were no granules and no differentiated layer of substance. This indicates that these granules and the layer of substance in which they are imbedded have but little if any functional relation with the contractile vacuole. The layer of substance is probably due to the action of the fluid in the vacuole on the adjoining cytoplasm.

Discussion

There is considerable confusion in reference to the ideas held by different investigators concerning the designation of the structure and the function of the granules and crystals in the cytoplasm of the protozoa.

Bütschli (1881, Abt. 1, S. 103) maintains that there are in fresh-water rhizopods and other protozoa, numerous highly refractive bodies ("Körner") which vary greatly in size "von ausserster Kleinheit bis zu ziemlich ansehnlichen Dimensionen," and frequently appear in the form of rhomboid crystals, and that they are not composed of fat. He holds that these bodies are end-products of metabolism and he calls them "Excretkörnchen." He obviously, therefore, includes under this term various kinds of granules and crystals, that is, all which are highly refractive except those which are composed of fat.

Schewiakoff (1893) found in *Paramecium* small bodies he called "Exkretkörner." He studied these bodies in considerable detail and concluded that they consist of calcium phosphate and are optically active, "doppeltbrechend im polarisirten Lichte." He says (p. 32) "In Folge der Doppeltbrechung, welche bereits von Maupas ermittelt wurde, sind sie von anderen Plasmaeinschlüssen leicht zu unterscheiden." Schewiakoff's Exkretkörner therefore include only a portion of Bütschli's Excretkörnchen, in fact only a very small portion, for only a very small percentage of the granules and crystals in *Amoeba proteus* are optically active.

Metcalf (1910), as previously stated, maintains that there are on the surface of the contractile vacuole and scattered through the cytoplasm of *Amoeba proteus* small round granules. He calls them "microsomes," "Cytomicrosomes," or "excretory granules." Mast and

Doyle (1935) demonstrated that these granules are not optically active. They are therefore not like the Exkretkörner of Schewiakoff.

Taylor (1923, p. 266) says that certain granules which appear throughout the cytoplasm of *Euplotes* are apparently comparable with Metcalf's "cytomicrosomes" in *Amoeba* and *Opaline* (1910), Bütschli's "Exkretkörner" (1881), Maupas' "corpuscle refringents" (1883), and Schewiakoff's "Exkretkörner" in *Paramecium*.

Since Metcalf's cytomicrosomes differ radically from Schewiakoff's Exkretkörner, it is difficult to see how the granules in *Euplotes* can be comparable with both. Taylor does not say whether or not they are optically active but he calls them "crystaloidal, endoplasmic granules" and he says "I have never observed that they tend to aggregate about the contractile vacuole." They are therefore probably more nearly like Schewiakoff's Exkretkörner than Metcalf's "cytomicrosomes" or "excretory granules."

Taylor (p. 278) maintains that Khainsky (1911) observed granules pass through the walls of the food vacuoles into the cytoplasm and he says: "These crystalloids he [Khainsky] identifies with the Exkretkörner described at length by Schewiakoff. Khainsky thinks that eventually the granules are dissolved in the endoplasm and the solution is discharged to the outside by the contractile vacuole." I find that Khainsky presents evidence which indicates that granules pass from the food vacuoles directly into the surrounding cytoplasm, but I find nothing which indicates that he identified these granules with Schewiakoff's Exkretkörner or that he held that they are dissolved in the endoplasm and discharged through the contractile vacuole.

The fact that Bütschli designated the granules and crystals he observed "Excretkörnchen" clearly indicates that he held that they are involved in excretion. He says nothing, however, concerning this.

Schewiakoff maintains that the bodies he designated "Exkretkörner" dissolve in the cytoplasm and are excreted in fluid form by the contractile vacuole. He says (p. 55): "Es liegt demnach die Vermuthung nahe, dass die Exkretkörner im Protoplasma aufgelöst und im füssigen Zustande durch die kontraktile Vacuole nach ausen entleert werden."

Metcalf (1910) thinks that his "excretory granules" are "functionally connected with excretion." He gives nothing concerning the method involved, but his description of the relation between these granules and the action of the contractile vacuole shows clearly that he holds that they are not dissolved and discharged by the contractile vacuole. His conception concerning the method of function of these granules consequently differs radically from Schewiakoff's concerning the method of function of his Exkretkörner.

Taylor (1923, p. 277) says: "Vacuoles (contractile) in Euplotes make their first appearance either from the coalescence of other extremely minute vacuoles that easily escape detection, or from the transformation of small vacuoles which contain one or more granules ('Exkretkörner'), or they arise de novo." I can see no essential difference between the first possibility and the third.

Concerning the second possibilities, Taylor thinks the granules in question may pass from the food vacuoles into the cytoplasm in accord with Khainsky's observations referred to above, that vacuoles may thus form around these granules, that the granules may then dissolve, and that the fluid vacuoles thus formed may develop into contractile vacuoles; and he holds that if this obtains the origin of the contractile vacuole is traceable to the food vacuole. The fact, however, that the contractile vacuoles continue to operate normally for days in the total absence of food, that long after all vacuoles containing food and the granules which have passed from them into the cytoplasm have disappeared, practically proves that they do not originate in this way.

Concerning the third possibility Taylor says: "Now it is conceivable that here and there in this organism 'fluid centers' normally arise which contain in solution substances (e.g. catabolic products) of a kind and concentration sufficient to induce the gelation of the surrounding plasm, thereby forming de novo normal vacuoles."

This possibility is in full accord with Metcalf's views concerning the origin of the contractile vacuole, except that he holds that the beta granules ("excretory granules") are involved. The fact, however, that under some conditions the contractile vacuole forms in regions where there are but few if any of these granules and that it develops normally at times when there are practically none on the surface of it, demonstrates that if they are at all functionally connected with the origin or the development of this vacuole, it is only in a very minor way.

It may be said then that while it is highly probable that the contractile vacuole originates in minute localized aggregations of fluid in the cytoplasm, nothing is known concerning the factors involved.

There is no evidence concerning the function of the relatively viscous layer in which the beta granules are imbedded on the surface of the contractile vacuole.

Summary

1. The contractile vacuole in *Amoeba proteus* contains at the surface a well differentiated membrane about 0.5μ thick. Adjoining this

membrane on the outside there is usually a layer of substance in which numerous beta granules (Metcalf's "excretory granules") are imbedded. This layer is more viscous and heavier than the adjoining cytoplasm. It is usually about 3μ thick when the vacuole is maximum in size and it becomes thicker during contraction.

2. The beta granules around the contractile vacuole vary greatly in number and the layer of substance in which they are imbedded varies greatly in thickness, without any apparent variation in the function of the vacuole. These facts indicate that neither the granules nor the layer of substance is involved in the function of the contractile vacuole, at least not directly.

3. The differentiation of a layer of substance on the surface of the contractile vacuole is probably due to the action of the fluid in the vacuole on the adjoining cytoplasm.

4. There is much confusion concerning the ideas held by different investigators in regard to the nomenclature, the structure and the function of the various granules and crystals in the cytoplasm of the protozoa.

5. Bütschli's "Excretkörnchen" consist of various different kinds of granules and crystals found in protozoa. Schewiakoff's "Exkretkörner" are optically active bodies, nearly all of which, if not all, are crystals. Metcalf's "excretory granules" are not optically active. They are approximately 1μ in diameter and have staining properties like the mitochondria in other cells. The "Excretkörnchen" of Bütschli, the "Exkretkörner" of Schewiakoff and the "excretory granules" of Metcalf therefore differ radically in structure.

6. Bütschli says the "Excretkörnchen" are end products of metabolism but he says nothing concerning their elimination. Schewiakoff contends that the "Exkretkörner" are formed in the food vacuoles, pass out into the cytoplasm, dissolve there and are eliminated by the contractile vacuole. Metcalf holds that the "excretory granules" (Mast's beta granules) are permanent structures which function in excretion of substance by the contractile vacuole. Mast and Doyle maintain that they function in transporting substance through the cytoplasm.

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DURATION OF LIFE WITHOUT FOOD IN DROSOPHILA PSEUDOÖBSCURA

OLE LILLELAND

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California)

Race *A* and Race *B* of *Drosophila pseudoöbscura* have been distinguished because they produce sterile male hydrids when crossed. Morphologically the races appear identical, and there are but few physiological characteristics that differentiate them. Thus, Poulson (1934) found that Race *A* flies have a shorter period of development than Race *B* flies. Dobzhansky[1] (1935) has studied the fecundity of both races at different temperatures and showed that during the lifetime Race *A* deposits more eggs than Race *B* at higher temperatures (25° C.), while at lower temperatures (19°, 14°) the relations are reversed. At all temperatures used Race *B* begins to oviposit later than Race *A*. In these experiments Dobzhansky observed also some indications suggesting that the longevity of Race *A* is greater than that of Race *B*. The present study is an attempt to secure further information bearing on this fact. In my experiments the measurements of the duration of life have been made in the absence of food. The data are therefore not necessarily comparable with those of Dobzhansky.

METHOD

Pearl and Parker (1924) state that any study involving longevity must be so conducted as to eliminate any desiccation; otherwise, the results are due to desiccation and not to the inherent factors which determine the length of life. Furthermore, they state that any technique which permits a fly to obtain water or any liquid even in small drops becomes a food study and not a starvation study. In the present work, however, the interest centered in the reaction of the two races to varying humidities and temperatures and not purely in the length of life at one optimum condition. Undoubtedly at a humidity of 95 per cent the flies took moisture condensing on the side of the tube.

Five bottles each of Texas (A) and Seattle—6 (B) cultures were started on the twenty-ninth of May, each bottle containing 3 females and 5 males from stock that had been under laboratory conditions for some time. These were placed in the 19° (C.) cold room and transferred every 48 hours to fresh bottles, until four successive transfers had been made. Any cultures which showed a total number of pupæ under 100 or above 400 were discarded. On the fourteenth day of the culture's life, the paper was removed from the bottle and the pupæ were scraped from it, using a pair of small forceps. Any pupæ which were coated with food were discarded. The removed pupæ were placed in one-ounce vials, plugged with cotton and placed again at 19° to await the first hatching.

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[1] The writer wished to express his indebtedness and sincere appreciation to Dr. Dobzhansky.

Poulson (1934) found that the maximum hatching occurred between the hours of 6 and 10 A.M., excepting Race *B* females which emerged in greatest numbers between 2 and 6 A.M. Since a fair percentage of the latter also hatch between 6 and 10 A.M. these four hours were chosen as the most convenient within which one might obtain representative populations. Each day of the hatch, therefore, all vials were emptied of imagines at 6 A.M., and at 10 A.M. the flies which had emerged were etherized and placed in sterilized glass vials, 20 per vial, sexes segregated. Each vial was covered with a piece of medium muslin, held tight by a rubber band. The flies were allowed to recover from the effects of etherization before being placed in desiccators. Using Obermiller's (1924) table of vapor pressures over salt solutions, the following chemicals were used: concentrated H_2SO_4 for 0 per cent, KOH (fused) for 8 per cent, $K_2CO_3 \cdot 2H_2O$ for 43 per cent, and K_2SO_4 for 95 per cent humidity.

Table I

Duration of life (in hours) at densities of 5 and 30 flies per one-ounce vial. Temperature 19° C., humidity 8 per cent.

Race	Five per vial		Thirty per vial		Difference
	$M \pm m$	n	$M \pm m$	n	
Seattle—6 ♀ (B)	71.70±1.18	90	69.60±1.10	60	+2.10±1.61
Seattle—6 ♂ (B)	74.90±2.01	70	68.80±1.44	60	+6.10±2.45
Texas ♀ (A)	102.57±1.20	105	97.05±1.17	150	+5.52±1.67
Texas ♂ (A)	104.88±1.76	75	98.67±1.33	90	+6.21±2.19

After recovery the vials were numbered and placed upright in the desiccator, using a piece of white cardboard to hold the single row of vials close to the glass of the desiccator so that counts could be taken without removing the desiccator top.

RESULTS

An attempt was made to determine the length of life of *D. pseudoöbscura* at two population densities. Pearl and Parker (1924) found that, on food, densities from 35 to 45 flies per bottle were optimum for *D. melanogaster.* At lower and especially at higher densities a reduction of the life span was observed. The data for *D. pseudoöbscura* are presented in Table I.

TABLE II

Duration of life at two temperatures and four different humidities

		Humidity 0 Per cent		Humidity 8 Per cent		Humidity 43 Per cent		Humidity 95 Per cent	
	Race	*M* ± *m*	*N*	*M* ± *m*	*N*	*M* ± *m*	*N*	*M* ± *m*	*N*
Temperature, 19°	Seattle—6 ♀	70.38 ±1.91	60	77.64 ±1.62	120	94.28 ±1.01	238	101.60 ±1.90	51
	Sequoia—8 ♀	63.10 ± .755	60	65.12 ±1.77	161	81.00 ±1.28	180	104.25 ±1.39	48
	Texas ♀	85.76 ±1.10	60	97.20 ± .77	55	102.97 ± .69	396	127.60 ± .98	53
	Mara—3 ♀	67.45 ±1.14	129	85.80 ±1.93	130	100.68 ±1.47	139	110.52 ±2.02	60
	Seattle—6 ♂	74.0 ±1.39	60	74.40 ±1.14	131	99.10 ±2.415	210	92.40 ± .13	70
	Sequoia—8 ♂	66.75 ± .955	60	66.37 ±1.15	159	89.22 ±1.20	160	102.00 ± .86	40
	Texas ♂	74.46 ±1.87	60	91.32 ±1.06	100	104.93 ± .80	254	111.0 ±1.33	40
	Mara—3 ♂	73.57 ±1.02	110	87.00 ±1.88	97	99.84 ±2.10	120	113.10 ±1.90	40
Temperature, 24°	Seattle—6 ♀			53.52 ± .90	120	55.16 ±1.80	100	60.50 ±1.10	40
	Sequoia—8 ♀	35.46 ± .45	100	45.60 ± .733	80	57.08 ±1.37	80	65.10 ± .705	100
	Texas ♀	57.30 ± .945	80	63.50 ±1.06	100	73.84 ±1.45	60	85.80 ±1.67	40
	Mara—3 ♀	35.58 ± .498	100	50.88 ± .657	220	63.60 ±1.63	80	74.40 ±2.36	80
	Seattle—6 ♂			50.79 ± .98	120	54.68 ± .566	120	62.76 ± .115	40
	Sequoia—8 ♂	37.80 ± .54	60	43.84 ± .857	80	56.05 ±1.25	60	71.66 ± .945	80
	Texas ♂	59.52 ±1.36	60	62.57 ± .917	100	75.46 ±1.43	80	87.90 ±1.43	80
	Mara—3 ♂	38.40 ± .39	80	52.80 ± .622	200	71.55 ±1.36	60	76.83 ±1.65	60

The data presented in Table I tend to show that the lower density is more favorable for *D. pseudoöbscura* than the higher one. They show furthermore that the duration of life of Race *A* is greater than that of Race *B*. The latter conclusion must, however, be checked in several respects. First of all, the fact that one strain of Race *A* differs from one strain of Race *B* does not necessarily indicate that the difference is characteristically racial; it may be a property of the particular strains used. Moreover, a difference observed under one set of conditions may be obliterated or even reversed under other conditions. The longevity of the flies has therefore been studied at temperatures 19° and 24° C., and at humidities of 0 per cent, 8 per cent, 43 per cent and 95 per cent (Table II). Finally, a variety of strains of both races coming from different parts of their distribution area has been studied (Table III).

Among the strains used, Texas, Mara, Julian, Oaxaca, Olympic, Pavilion, and Taos belong to Race *A*, and Seattle, Sequoia, Campbell, Crater, Quilcene, and Quesnel to Race *B*. It can be seen from Tables

II and III that in general Race *A* tends to be more long-lived than Race *B*. Nevertheless, some intra-racial variation is observed. Thus, the longevity of the Texas strain is greater than that of the Mara strain; the Quesnel strain is superior to other Race *B* and to some Race *A* strains. It may be noted that at 95 per cent and 43 per cent humidity Race *A* strains are in general superior in longevity to Race *B*. At lower humidities, especially at 0 per cent, a greater degree of overlapping between races is observed. Qualitatively, the effects of humidity and temperature are identical for all strains studied. The duration of life is greater at the lower temperature (19°) than at the higher one (24°), and at higher humidities than at the lower ones.

TABLE III

Duration of life in nine strains of *D. pseudoöbscura* at 24° C. and three different humidities

	Humidity 0 Per cent			Humidity 43 Per cent		Humidity 95 Per cent	
	Race	*M* ± *m*	*N*	*M* ± *m*	*N*	*M* ± *m*	*N*
Race *B*	Campbell—4 ♀	40.13± .770	90	48.85±.615	80	68.57±1.40	80
	Crater—2 ♀	38.09± .628	60	46.80±.514	60	61.86±1.05	60
	Quilcene—4 ♀	40.00± .740	90	47.04±.648	60	66.24±1.68	40
	Quesnell—5 ♀	46.91± .655	60	56.12±.727	68	69.43±1.585	60
	Campbell—4 ♀	41.02± .634	103	51.64±.605	80	66.62±1.54	60
	Crater—2 ♂	38.10± .715	60	50.00±.595	60	62.15±1.095	82
	Quilcene—4 ♂	38.84± .500	120	43.08±.468	60	57.30±1.49	60
	Quesnell—5 ♂	47.07± .475	60	55.41±.495	80	59.10±1.295	60
Race *A*	Julian E—6 ♀	53.02± .770	80	63.72±.755	60	83.62±1.96	60
	Oaxaca—4 ♀	43.08± .707	85	50.64±.741	60	90.54±1.86	80
	Olympic—2 ♀	46.62± .684	80	61.58±.787	40	92.05±1.69	80
	Pavillion—5 ♀	49.25± .705	89	66.37±.455	100	88.88±1.54	60
	Taos—1 ♀	49.89±1.18	70	56.40±.800	60	70.10±1.54	80
	Julian E—6 ♂	47.02± .655	100	60.00±.482	80	76.44±2.21	60
	Oaxaca—4 ♂	45.32± .644	100	56.57±.616	60	90.73±2.41	60
	Olympic—2 ♂	46.85± .632	90	65.43±.880	40	92.93±1.68	80
	Pavillion—5 ♂	50.00± .806	60	64.87±.545	80	89.73±2.01	40
	Taos—1 ♂	51.78± .955	60	61.60±.641	80	64.56±2.00	60

The two races of *D. pseudoöbscura* differ in their geographical distribution. Race *B* is restricted to the northern part of the Pacific Coast, while Race *A* lives much further eastward and southward than Race *B* (Dobzhansky, 1935, 1937). As far as our data show, the effects of humidity and temperature on the duration of life in the absence of food have little bearing on the geographical distribution of the two races. Further studies, especially those on the duration of life in the presence of food, may conceivably throw more light on this problem.

SUMMARY

1. In the absence of food, the duration of life of *Drosophila pseudoobscura* is greater at lower temperatures, greater humidities, and lower population densities studied.

2. With temperature, humidity, and population density being kept constant, Race *A* lives longer than Race *B*. These differences between the races is more pronounced at higher than at lower humidities.

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THE LIFE HISTORY OF A TREMATODE (LEVINSENIELLA CRUZI?) FROM THE SHORE BIRDS (LIMOSA FEDOA AND CATOPTROPHORUS SEMI-PALMATUS INORNATUS)[1]

(Contributions from the Scripps Institution of Oceanography New Series, No. 19).

R. T. YOUNG

(*From the Scripps Institution of Oceanography, La Jolla, California*)

Introduction

Lebour (1911) has described a cercaria (*C. ubiquita*) from British snails (*Paludestrina stagnalis, Littorina obtusata* and *L. rudis*). She found "Cercariæ exactly corresponding with *C. ubiquita*, but without the glands, inside the tissues of *Carcinas mœnas*" and similar metacercariæ were found by her in *Cancer pagurus*, the third host in the cycle being presumably the herring gull (*Larus argentatus*). The same or a closely related larva has been found by Stunkard (1932) in *Littorina rudis* and *L. littorea* at Roscoff, France, and Rees (1936) has observed similar larvæ in these snails and in *L. obtusata* at Aberystwith, England. The adult form of these larvæ is supposed to be some species of *Levinseniella*, but thus far the various steps in the life history have not been definitely established.

In a study of the trematodes of the La Jolla region on which I am engaged I have found a stylet cercaria infesting the snail *Olivella biplicata* which differs in some particulars from those of Lebour and Stunkard, and whose probable adult form I have located in the shore birds (*Limosa fedoa* and *Catoptrophorus semipalmatus inornatus*), while the metacercaria parasitizes the sand crab (*Emerita analoga*).

I will first describe the cercaria, the metacercaria and the adult worm, and then give the evidence on which my conclusions regarding the life history are based.

The Cercaria

The cercaria (Fig. 1) is one of the spineless xiphidio-cercariæ, possessing a stylet from 24 to 32 μ long, and a set of eight unicellular penetration (?) glands, but no pigment spots. In fixed specimens the body averages 165 × 46 μ and the tail 124 μ in length. It has an oral sucker 34 μ in average diameter, but no acetabulum, or gut. The excretory bladder is bifurcate, but further details of the excretory system are not evident.

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[1] The following study has been conducted at the Scripps Institution of Oceanography, to whose directors, Dr. T. Wayland Vaughan and Dr. H. U. Sverdrup, and other members of the staff I am indebted for many courtesies.

Perhaps the most interesting feature of the larva are the penetration (?) glands, which are in two sets, an anterior set of four larger, and a posterior one of four smaller glands. Their arrangement is shown in Fig. 1, where they are designated by numbers 1–8 respectively. Each gland has a duct which follows a more or less sinuous course depending on the state of contraction of the worm, and which opens at the anterior border of the sucker. Lebour states that there are but four ducts for the group of glands in her larva, but their close apposition and sinuous course renders their individual identification difficult and might easily lead to confusion about their actual number. My specimens, when stained with neutral red, show clearly one duct for each gland. Both glands and ducts stain intensely in this stain.

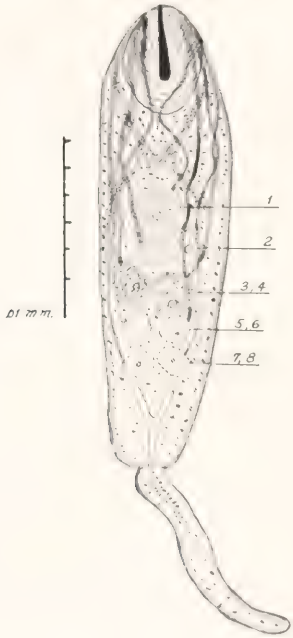

FIG. 1. Cercaria showing stylet, sucker, nerves, excretory bladder and glands (1–8). For description of latter, see text. Outline with camera, details free-hand.

These glands differ markedly; not only in size, but also in the physical and apparently chemical character of their contents. Glands No. 1 and 2 are finely granular and do not ordinarily take an eosin stain. Occasionally Nos. 1 and 2 stain lightly in eosin but much less intensely than Nos. 3 and 4. Numbers 3 and 4 are also finely granular, though the granules are perhaps a trifle coarser than in Nos. 1 and 2, but they take the eosin stain intensely. Glands 5–8 stain very lightly. They contain only a few eosinophile granules which are scattered through the meshes of a rather open reticulum, so that the whole cell appears as an open space in the sections with what is usually a pycnotic, heavily stained nucleus near the center. The nuclei in all eight glands, however, vary widely, sometimes being pycnotic and densely stained, and at other times vesicular and so lightly stained as to be scarcely visible. Especially is the latter true of glands 1 and 2. That these differences are real and not artificial is indicated by the fact that they may occur in neighboring larvæ in the same sporocyst. These structural and staining differences are evidence of functional differences in the two sets of cells 1–4 and 5–8 respectively. It is probable that each set elaborates a different secretion, for were the differences in structure

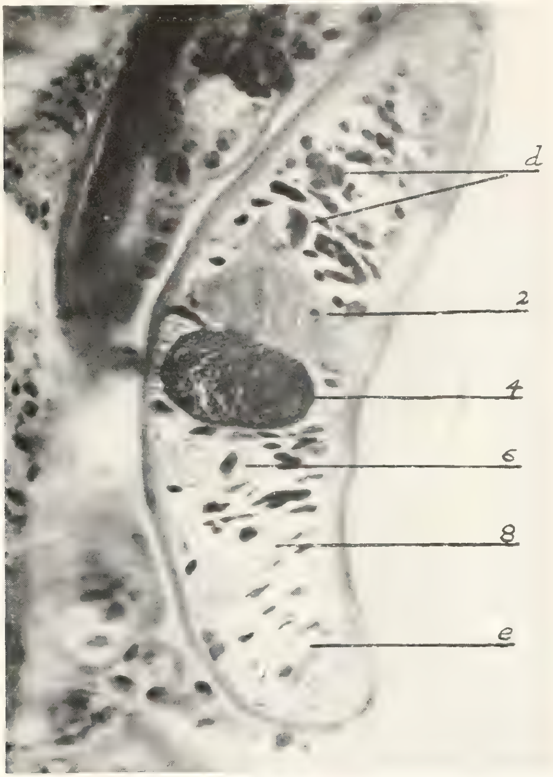

FIG. 2. Photomicrograph of long section of cercaria showing glands 2, 4, 6 and 8 (see Fig. 1 and text). *d*, ducts; *e*, excretory bladder; × 860.

and staining capacity due to different stages in the elaboration of the *same* secretion the two sets of cells should vary in different individuals. But this is apparently not the case; each set is distinct in every cercaria. The difference in eosinophily between cells 1, 2, and 3, 4 respectively is probably explicable by differences in the stage of development of the same secretion, but this will not explain the difference between sets 1–4 and 5–8.

Stunkard (*loc. cit.*, p. 337) says of his larva that "the cells do not stain with neutral red, but the secretory granules are clearly visible . . . (which granules) stain a deep blood red in the terminal portion of the ducts and frequently accumulate there to form enlargements," while Rees (*loc. cit.*, p. 622) says that "only the two anterior pairs (of cells) and their ducts . . . (stain) a deep red. The remaining cells and their ducts have never been observed to take up the intravital stain." All eight cells in my larva stain uniformly in neutral red, the differences appearing in the material stained with eosin.

It is difficult to harmonize these various observations with one another. They *may* indicate specific differences in the cercariæ themselves but it is not impossible that they are due to functional differences in the glands at different times and under different conditions. Possibly they are the effect of different hosts.

The infestation of the snail is considerable, 6 out of 98 specimens examined being infested.

The shedding of the cercariæ appears to be a seasonal matter, although other, as yet obscure factors may affect this. In February and March, 1935, cercariæ were easily obtained by isolating snails from the beach near the Scripps Institution, whereas from November, 1936 to January, 1937 isolation of several hundred snails produced no cercariæ. In May and June of 1937, a few cercariæ were obtained from several hundred snails, but in July and August, 1937, isolation of several hundred snails failed to produce any. That some of the apparently sterile snails were infested, however, was proven by dissection of several specimens. Lebour (*loc. cit.*) also has observed that snails are free from *C. ubiquita* in winter, while their walled cysts (metacercariæ) are present in crabs only during spring and summer and early autumn. I have not made sufficient study of this matter, however, and hence cannot define definitely the seasonal cycle of the life history, if such exists, which appears to be the case.

THE METACERCARIA

The cercariæ in the crab lack tails, but whether these are lost before or after entrance I cannot say. The former appears more likely, however, as the tails are frequently detached from the cercariæ while swimming. Soon after entrance the glands and ducts become broken down and are identifiable only as irregular masses which still stain in neutral red. Within two or three days the cercaria secretes a cyst within which it curls up and begins its development into the metacercaria.

This naturally varies in size with age, the older ones reaching a diameter of 0.3 mm. with a cyst wall about .025 mm. thick. It is very slightly ovoid in shape and is found in the connective tissue of the crab, chiefly among the liver tubules. Practically every crab examined, in the La Jolla region, over 6 mm. in length is infested. I have not determined the maximum number of cysts which one crab may carry, but it is probably several dozen.

The Adult

The adult worm agrees closely in all important details with the description of the genus *Levinseniella* as given by Jägerskiöld (1900). The outline is narrower in front and broader behind, and when much expanded the body may be slightly constricted just in front of the acetabulum. This latter is located about two-thirds the length of the body from the anterior end. The anterior two-thirds of the body is spinose, the spines gradually diminishing in size and disappearing at about the level of the acetabulum.

Judging from the figure (unlabeled) and the description (in Portuguese) of *L. cruzi* by Travassos (1920) my specimens belong to this species, although the difference in the orders of the hosts (*Anseres* and *Charadriformes*) and in locality (California and Rio de Janeiro, Brazil) render this uncertain. As shown in Table I, the dimensions of my specimens correspond rather well with those of *L. cruzi*,[2] the chief differences being in the size of the oral sucker, which in my specimens averages 0.061 mm. in diameter while in the former it averages from 0.092 to 0.113 mm., and in the length of the ceca (0.134 and 0.163 to 0.241, respectively).

Perhaps the most striking feature of agreement between the two is in the size of the eggs, which average larger in both than in any other species known to me. The average of 24 eggs from my specimens fixed in aceto carmine is 0.036 by 0.017 mm., while in *L. cruzi* they average from 0.021 to 0.035 by 0.012 to 0.014. When fairly extended in life my specimens range from 0.55 to 0.9 mm. in length, while *L. cruzi* ranges from 0.609 to 0.713 mm. While there thus appear to be certain minor differences between my worms and those of

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[2] Presumably measured from fixed material although Travassos does not so state.

TABLE I

Table I showing average measurements in μ of fresh and preserved specimens, with the number of specimens averaged for each measurement in parentheses. With the exception of five eggs, all preserved specimens were fixed in aceto-carmine. There is some overlap in the two sets, i.e. a few specimens were measured first in the fresh, and then in the preserved condition. Column 3 shows the measurements of *L. cruzi* from Travassos (*loc. cit.*).

	Fresh	Preserved	*L. cruzi*
Length	692 (14)	676 (15)	609–713
Width	193 (14)	303 (15)	222–313
Oral sucker	61 (12)	61 (14)	92–113
Pre-pharynx	37 (3)	36 (5)	14–56
Pharynx	33×21 (8)	32×23 (9)	35×53–21×35
Post-pharynx	168 (4)	170 (10)	63–120
Ceca	112 (7)	134 (10)	163–241
Acetabulum	54 (15)	55 (15)	56–71
Acetab. distance (from ant. end)	485 (9)	457 (9)	
Penis diameter	23 (9)	23 (9)	
Seminal vesicle	67×39 (5)	67×38 (8)	
Ovary	74×49 (12)	71×54 (15)	56–92
Right testis	97×58 (12)	93×69 (14)	
Left testis	90×61 (12)	89×65 (14)	
Egg		36×17 (24)	35×14–21×12

Travassos, they are insufficient in my judgment for separating them specifically, and I am therefore, in spite of the difference in hosts and locality, provisionally assigning my specimens to his species.

The Life History

The evidence upon which my conclusions regarding the life history of this worm are based is briefly as follows:

(1) the presence in the sand crabs of stylet cercariæ, and of all stages in the developing metacercariæ from that of the cercaria surrounded by a very thin cyst, but still having the stylet and remnants of the penetration (?) glands to those of the trematode, which resembles the adult in every way save for the presence of eggs in the uterus;

(2) the experimental infestation of crabs with cercariæ;

(3) the presence in the birds of adult worms which resemble in every way the excysting metacercariæ, save for the presence of eggs in the uterus, and the presence of both encysted and excysted metacercariæ in the birds' stomachs after they have fed on sand crabs.

There is apparently in nature a universal infestation of all crabs more than 6 mm. long. I have not recorded the number of crabs which I have examined (50–100), all of which were infested. It is probable that infestation begins when the crabs are about 5 mm. long as I have found specimens of this size containing cercariæ. Until they reach a length of at least 5 mm. the crabs are less frequently infested. Of 30 specimens of this size or less which I examined in nature only 8 contained cercariæ. It is evidently a matter of age, and consequently length of time of exposure, which determines the infestation, for the larger specimens are more heavily infested than the smaller ones, and very young crabs are susceptible to experimental infestation. The season of the year is possibly also an important factor, for, as already shown,[3] the deposition of cercariæ by the snails may be dependent on the season.

I have performed three sets of experiments in an attempt to demonstrate the transfer of cercariæ from snail to crab, the results of which are summarized in Table II.

In the first of these a number of cercariæ were obtained from snails which shed them naturally, transferred to a finger bowl with ten crabs from 4 to 5 mm. long, and left for from three to four days, after which, examination of the latter showed that seven out of eight (all of which were examined at the time) contained from one to four cercariæ each while the remaining crab which was dissected 42 days later was negative.

[3] See page 322.

As a control 15 crabs collected on the beach within four weeks of this experiment gave a ratio of 5 infested to 10 non-infested specimens.

An experimental infestation of seven out of nine young crabs, while 10 of 15 in nature were uninfested, has a probability, on the basis of chance alone, of .03333.[4]

A second group of experiments consisted in holding for one or two days 18 young *Emerita* in finger bowls, together with cercariæ which had just been shed by snails. Seven of these were subsequently found to be infested. In the control for this experiment, out of 20 young crabs none were infested. In this experiment and its control the crabs were kept for several days previously in running sea water which was filtered through several inches of sand, thereby eliminating the likelihood of an accidental infestation. The probability of this result occurring on the basis of chance alone is .0021.[4] In explanation of the low ratio of infestation (7 : 18) in this series of experiments, as compared with the high ratio (7 : 9) in the former series, it may be said that the number of cercaria per crab was much smaller in the latter than in the former series.

TABLE II

Showing results of experimental infestation of *Emerita* with the cercaria of *Levinseniella cruzi* (?)[5]

Series 1		Series 2		Series 3	
Experiment *Emerita* exposed to cercariæ		Experiment *Emerita* exposed to cercariæ		Experiment *Emerita* exposed to *Olivella*	
Positive	Negative	Positive	Negative	Positive	Negative
7	2	7	11	17	18
Control *Emerita* collected on beach		Control *Emerita* in filtered sea water		Control *Emerita* in unfiltered sea water	
Positive	Negative	Positive	Negative	Positive	Negative
5	10	0	20	6	40

In a third series of experiments *Emerita* were held from one to several days in jars[6] containing infested snails for comparison with crabs held in running sea water. The result of this experiment was an infestation of 17 out of 35 crabs in the experimental jars compared with 6 out of 46 in the control. The probability of this result on a purely chance basis is .0003.[7]

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[4] Computed from McEwen (1929).

[5] In determining the presence or absence of parasites in the crabs the factor of length of examination is essential. I adopted a minimum of 12–15 minutes for the smaller (5–6 mm.) specimens and of 15–20 for the larger (10–15 mm.) ones.

[6] Some of these were supplied with running sea water. In others the water was standing, but aerated.

[7] Computed from McEwen (1929).

In evaluating this series of experiments it should be observed that in many of them only a very meagre number of cercariae were furnished a considerable number of crabs in a volume of from two to eight liters of water, hence the chance of any individual crab receiving a cercaria was rather small. The infestation of a considerable number of crabs in the controls (6 in 46) is difficult to explain. Very rarely cercariae occurred in the laboratory water supply, but straining through cotton a small stream of water from a tap for about forty hours produced no cercariæ, so that their chance occurrence in the control aquaria can hardly be explained thus. In many cases the control aquaria were standing on the same table with jars which contained possibly infested snails and it is possible that cercariæ from the latter may have infested the controls. In one case in particular a control jar of *Emerita* collected on August 17, 1937 contained recently infested crabs until August 28, during which time a jar of snails was standing on the same table; but straining the water from this jar through fine bolting cloth on this date failed to produce any cercariæ. On August 30 and September 1 after removal of this jar an examination of ten crabs from this control revealed no recent infestations.

The source of the occasional infestation of the controls therefore remains uncertain, but in any case the difference in the infestation ratios between experiments and controls in this series is highly significant.

Taken collectively the experiments give a result of 31 infestations out of 62 tests, while the controls give 11 out of 81 tests; and these include 5 out of 15 exposed to natural infestation on the beach. This result, on the basis of chance alone, has a probability of .0000013.[8]

Larvæ similar to those in the sand crabs occur in the fiddler crabs (*Uca crenulata*) from Mission Bay, an inlet of the sea near La Jolla. Doubtless, many other crabs of the vicinity harbor the same or related larvæ, for Lebour (*loc. cit.*) and Stunkard (*loc. cit.*) in Europe have found related metacercariæ in several species of crabs.

The extent of infestation of the willet and godwit by this parasite is rather limited. Only toward the end of this study did I differentiate between this species and two other related ones so that I cannot give any figures on the percentage of infestation. However, the former is usually, if not always, greatly outnumbered by what appear to be two other species of *Levinseniella*. In at least one case, that of a marbled godwit, it was apparently absent, but time did not permit an examination of the entire gut of this bird so that possibly a few specimens escaped observation.

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[8] Computed from McEwen (1929).

McMullen (1935) gives some data which are of interest in this connection. In experimental infestations of *Amia calva* with *Macroderoides typicus* he found the fish heavily infested a few days after feeding infested material, but after several months the infestation was only slight. Thus one fish examined after 22 days contained by actual count at least 1392 flukes, while another fish examined 202 days after infestation contained only 33. He believes that "This enormous loss . . . was probably . . . due to crowding" (*loc. cit.*, p. 375). And Sarles and Stoll (1935, p. 290) state that "cats . . . carrying natural infestations of *Toxacara cati* were found to possess a uniformly high degree of resistance against attempts to superimpose infection with this ascarid." What limits the degree of infestation in wild animals, many of whom, as is the case with these shore birds, have unlimited opportunity for acquiring it, is as yet an unsolved problem. It may be, as suggested by Stunkard (1930) and Stoll (1929), analogous to the development of immunity by animals to bacterial infection. In the case of *L. cruzi* and the shore birds it is possible that the usually abundant infestations with other species limits the number of the former. It does not, however, apparently interfere with extensive infestation by a *Maritrema sp.* which the birds probably receive from the sand flea (*Orchestoides*), which forms a considerable part of their diet.

In trying to follow the life history of this worm I was at first misled by finding excysted individuals in the surf perches (*Embiotoca*, *Abeona*) after the latter had eaten sand crabs. It was only after performing several experiments that I discovered that the infestation of the fish is only temporary. It is rather remarkable, however, that a parasite of a homoiothermal animal should excyst and exist, even for a brief period in a poikilothermal form. My observations on this point are not solitary, however, for Linton (1928) reports *Levinseniella adunca* from a sanderling (*Crocethia alba*) which "appeared to be identical with a species found in the toad fish (*Opsanus tau*)" (*loc. cit.*, p. 21).

The last link in the life history of this worm is not yet made. In spite of repeated fecal examinations of both wild and captive birds, I have not succeeded in finding either eggs or miracidia. The eggs in utero appear never to have developed beyond an early cleavage stage. I have one probable record of a miracidium in the snail, but as my observations were very brief, the organism being lost in transfer to a slide, I am not able to assert positively that it *was* a miracidium, much less to determine whence it came. There must, however, be some period when the birds are shedding eggs or miracidia. This I hope subsequent study will reveal.

Summary

The life history of a trematode (*Levinseniella cruzi?*) from the shore birds (*Limosa* and *Catoptrophorus*) and the structure of its cercaria have been described in some detail. The probable life history is as follows:

The miracidium has not yet been certainly observed. The cercaria, which belongs to the ubiquita group of Lebour, inhabits the snail, *Olivella biplicata*. From here it passes to the sand crab, *Emerita analoga*, which latter, when eaten by the birds, infests them with the adult trematode. The degree of infestation of the snail is considerable, but the cercariæ are shed infrequently, possibly at definite seasons.

Practically all crabs over 6 to 7 mm. in length are infested, but for some as yet undetermined reason, the infestation of the birds is light.

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THE EFFECTS OF ULTRA-VIOLET RADIATION OF λ2537A UPON CLEAVAGE OF SEA URCHIN EGGS[1]

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The effect of ultra-violet radiation upon the division rate of various cells has been the subject of a number of investigations, but the results are not in complete agreement, some investigators claiming acceleration (e.g., Coblentz and Fulton, 1924; Hinrichs, 1928; Alpatov and Nastjukova, 1933), others retardation (*e.g.*, Hertel, 1905; Gates, 1929; Oster, 1934; Chase, 1937), still others acceleration or retardation depending upon the wave-length and dosage (*e.g.*, Bovie and Hughes, 1918; Hughes and Bovie, 1918; Hutchinson and Ashton, 1929).

The disagreement may be more apparent than real, the results depending upon the wave-length of the ultra-violet, the dosage and the organism used. Quantitative data are needed to throw further light upon the problem. The following paper is an attempt to gather such data on the effects of one wave-length, 2537A, upon cleavage of sea urchin eggs, this material being chosen because the self-contained food supply greatly simplifies control of the environment. Work is planned at each of the other wave-lengths of ultra-violet light represented in the spectrum of the quartz mercury arc.

MATERIALS AND METHODS

The sea urchins (*Strongylocentrotus purpuratus* Stimpson) were collected during the winter breeding seasons of 1935 and 1936 at Moss Beach and Pacific Grove, California. Eggs were obtained after natural spawning or by excision of the ovaries and from 25 to 100 eggs were placed in each 1″ watchglass containing 3/4 cc. sea water. The sperm suspension was determined each time by tests, successive dilutions being made until 100 per cent fertilization was achieved without overinsemination, and since almost all eggs cleaved normally, polyspermy was probably rare. Only eggs in which practically 100 per cent showed fertilization membranes within two minutes after insemination were used in this research.

In the case of eggs kept at 14–16° C., the first cleavage occurred in about two hours following insemination, the second after another

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[1] This work has been supported in part by funds granted by the Rockefeller Foundation.

hour, and the successive cleavages to the 64-celled stage at about hourly intervals. Within 20 hours after insemination the actively swimming blastulæ had been formed and in about another 24 hours gastrulation was complete, having begun some 12 hours earlier. Plutei formed within 4 days after insemination although the arms did not begin to elongate for another 24 hours. Since controls showed normal development to the pluteus, the conditions were considered satisfactory for the investigation.

For the first series of studies a mercury-argon discharge tube which emits about 88 per cent of the total output in the visible and ultraviolet at λ2537A (Coblentz, 1931; Leighton and Leighton, 1935) was employed since its high intensity enables one to give an effective dose of radiation in a short time and renders feasible certain experiments otherwise impossible. The intensity of the radiations, after screening out the infra red rays by a suitable water filter, was shown by thermopile measurements to be relatively constant for the period of investigations.

For experiments where pure light of λ2537A was needed the radiations from a water-cooled quartz mercury arc were passed through a natural quartz monochromator and the light of the desired wavelength was focused on the quartz cell containing the eggs. The apparatus used was in general similar to that previously described (Giese and Leighton, 1935).

The line thermopile (type described by Leighton and Leighton, 1932, p. 1884) used in series with a D'Arsonval H.S. galvanometer, was calibrated against Bureau of Standards Lamps C-211 and C-212. The thermopile factor for λ2537A was calculated to be 24.10 ergs/sec./cm. galvanometric deflection.

Experimental

Irradiation of Eggs Just Before the First Cleavage

In the first series of experiments the eggs which had been inseminated 90 minutes previously were irradiated for 1, 2, 4, 8, 16, 32, and 64 seconds at a distance of 31 mm. from the center of the mercury argon tube, and for 1, 4 and 16 seconds at a distance of 248 mm. from the center of the tube (to give exposures approximately equivalent to 1/64, 1/16, 1/4 second). In one series exposures approximately equivalent to 1/8 and 1/32 second were also given. Examinations were made at intervals of a half hour or an hour, depending upon circumstances, and the stages in development recorded. A typical set of data from a series of 3 experiments is plotted in Fig. 1.

It is clear from Fig. 1 that delay in cleavage is proportional to dosage and that even 1 second irradiation is sufficient to delay development for a long time, 1/4 second for a slight period of time. However, the protoplasm of the egg hastily repairs injury from lesser dosages, for example, the rate of cleavage of eggs irradiated 1/8, 1/16, 1/32 and 1/64 second was at no time lower than that of controls. But in no case was an increased rate of cleavage observed.

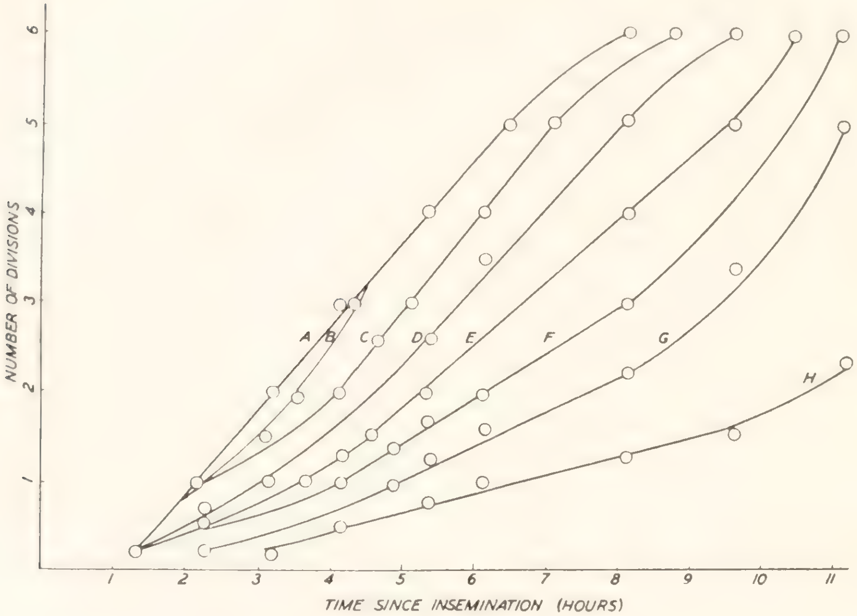

FIG. 1. Retardation of cleavage by radiation.

A. Control.
B. One-fourth of a second dosage.
C. One second dosage.
D. Two seconds dosage.
E. Four seconds dosage.
F. Eight seconds dosage.
G. Sixteen seconds dosage.
H. Thirty-two seconds dosage.

While many of the eggs irradiated for 4 or more seconds developed normally later, in most cases a retardation was observable even at later stages in development. Thus in Table I it will be noted that while 22 hours after insemination normal free-swimming blastulæ were formed from all eggs except those irradiated 4 seconds or more, those developed from eggs irradiated 4 and 8 seconds were still within the fertilization membranes. Even 32 hours after insemination gastrulation in these eggs was just beginning when the controls and those given smaller doses had completed invagination. Ultimately all the above eggs, even those irradiated as long as 8 seconds, gave rise to gastrulæ normal to all appearances.

TABLE I

Later development of eggs irradiated ninety minutes after insemination at the dosages indicated

Dosage in seconds	22 hours after insemination	32 hours after insemination	46 hours after insemination
1/64, 1/32, 1/16, 1/8, 1/4 and control	Normal free-swimming blastulæ	Normal gastrulæ	Beginning of gut differentiation
1	Normal free-swimming blastulæ	Normal gastrulæ	Gut not yet differentiated
2	Normal free-swimming blastulæ	Normal gastrulæ	Gut not yet differentiated
4	Normal blastulæ but still within membranes	Blastulæ with beginning of invagination	Early gastrulæ
8	Normal blastulæ but still within membranes	Blastulæ with beginning of invagination	Early gastrulæ
16	Mostly abnormal motile balls. Some nonmotile blastulæ.	Mostly abnormal. Show delayed gastrulation.	As before
32	Coagulated cells	—	—
64	Coagulated cells	—	—

In some series (5 trials) of eggs irradiated 16 seconds a fair proportion of the eggs formed blastulæ and gastrulæ; in other series of eggs so irradiated cleavage resulted in a mass of motile cells which persisted without differentiation for as long as observations were made. After dosages of 32 seconds eggs developed into masses of undifferentiated cells which never became motile and early appeared opaque. Dosages of 64 seconds apparently inhibited even one division, though in some cases cleavage did occur; more often the attempt at cleavage was abortive, the apparent blastomeres failed to separate, often fused and then cytolized.

Differential Susceptibility of Eggs at Different Stages

It would be of interest to compare the susceptibility of unfertilized eggs, eggs just inseminated, eggs well after insemination, and eggs in the first cleavage. First of all, however, it was necessary to determine whether the unfertilized egg would become activated with the dosages used. Three series of 3 trials each were therefore made with 1/64, 1/16, 1/4, 1, 16, 64, and 256 seconds of irradiation. In no case was a normal fertilization membrane formed without insemination, nor was division apparent. The dosages used do not, then, activate the eggs to artificial parthenogenesis, but experiments showed that when the dosage was not too great, eggs so irradiated when inseminated would develop normally.

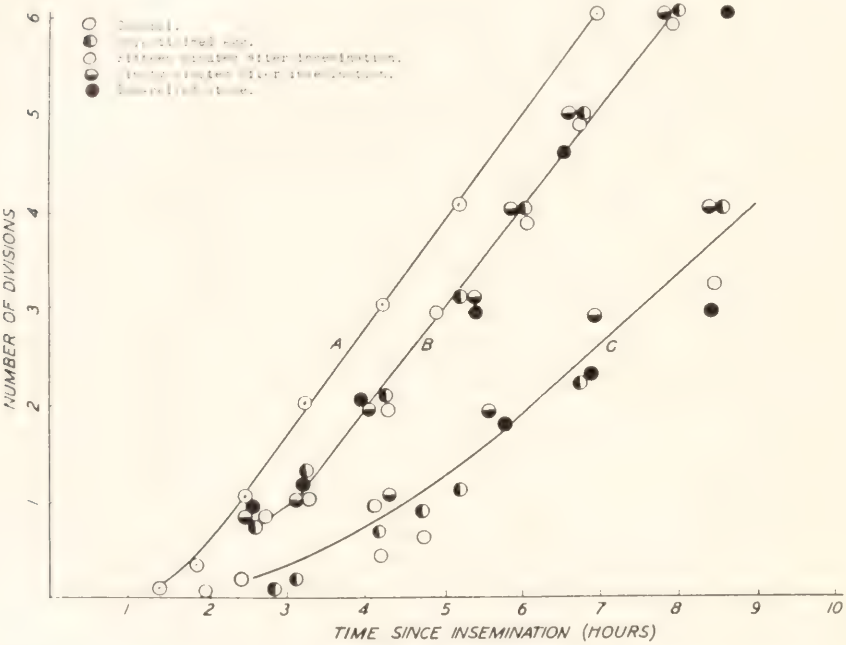

FIG. 2. Differential susceptibility to radiation of eggs at different stages of development.

A. Control.
B. One second dosage.
C. Sixteen seconds dosage.

Three series of experiments (3 each) were now performed on eggs in the following stages: (1) unfertilized, (2) 15 minutes after insemination, (3) 90 minutes after insemination and (4) the two-celled stage with dosages of 1, 4, 16, and 64 seconds of irradiation. The results, excepting those for eggs irradiated 4 and 64 seconds, are given in Fig. 2. It is readily observed that there is no very great difference of susceptibility of these stages, one second of irradiation reducing the cleavage rate about equally in all cases (4 seconds reducing the rate still more, but omitted from the graph to avoid confusion), 16 seconds much more. From Fig. 2 it may be observed that there is a latent period before the effects appear. This is particularly so for all stages irradiated for one second and for the 2-celled stage irradiated at all dosages. The eggs irradiated 64 seconds gave but few abortive cleavages when irradiated before insemination or 15 minutes after insemination. When irradiated for 64 seconds, 90 minutes after insemination, most of the eggs passed into the 2-celled stage, and then cleaved abortively. Apparently at the time of irradiation the mechanism of cell division was already in full swing and could not be stopped. Of eggs irradiated for 64 seconds just after the first cleavage only a small proportion continued to divide and these only for a short time afterwards.

As in the previous experiments retardation was not only obvious during early cleavage but also at later stages, for when controls had developed into free-swimming blastulæ, some of the irradiated eggs had developed into non-motile blastulæ, and when controls had gastrulated, some of those irradiated had only begun to gastrulate. The data for later development are given in full in Table II.

Three sets of eggs in the 2-celled stages were irradiated with dosages equivalent to 1/4, 1/16, and 1/64 seconds, but in *no* case was the cleavage rate greater than that of the controls—in fact, there was a slight delay when the dosage of 1/4 second was given.

Effect upon the Medium

When the mercury-argon discharge lamp is in operation, ozone in readily detectable quantities is produced by the action of the short ultra-violet (1849A) on the oxygen of the air. To determine whether this was dissolving in the medium and causing retarded development in the experiments reported above, sea water was irradiated for 64 and 256 seconds, then eggs were added and the development compared with the controls (5 experiments, 256 seconds; 10 experiments, 64 seconds). Unfertilized eggs, eggs 15 and 90 minutes after insemination and 2-celled stages were used. In all cases there were no signs of retardation, cleavage in all cases being comparable to the controls. The retarded cleavage of eggs irradiated with the mercury-argon tube is apparently due to the absorption of the ultra-violet radiation by the eggs and not to an effect upon the medium, and it is probably due practically entirely to radiation of λ2537A, since the only lines of significant intensity in the mercury-argon tube spectrum are 3130, 3660, and several visible lines (4050, 4358, 5461, 5790A), all of which are relatively inert.

TABLE II

Later development of eggs irradiated at various stages in development

Dosage in seconds	Irradiated at [illegible] stage in development	21 hours after insemination	Percentage of blastulæ abnormal	27 hours after insemination	45 hours after insemination	No. of eggs used
1	Unfertilized egg	10% non-motile blastulæ	0.0	All motile	Normal gastrulæ	155
	15 min. after insemination	4% non-motile blastulæ	0.0	All motile	Normal gastrulæ	271
	90 min. after insemination	5% non-motile blastulæ	0.5	All motile	Normal gastrulæ	198
	2-celled stage	8% non-motile blastulæ	1.0	All motile	Normal gastrulæ, smaller than controls	188
4	Unfertilized egg	10% non-motile	0.9	All motile	Normal gastrulæ, smaller	346
	15 min. after insemination	77% non-motile	0.0	All motile	Normal gastrulæ	121
	90 min. after insemination	85% non-motile	11.6	All motile	Normal gastrulæ smaller than controls	104
	2-celled stage	99% non-motile	7.6	All motile	Normal gastrulæ, few incompletely invaginated	144
16	Unfertilized egg	All non-motile	0.0	Almost all non-motile	Some abnormal; most smaller than controls	250
	15 min. after insemination	All non-motile	13.7	20% motile	16% normal gastrulæ. Others abnormal morulæ	257
	90 min. after insemination	All non-motile	24.0	76% motile	87% gastrulate, small, somewhat abnormal	175
	2-celled stage	All non-motile	30.8	5% motile	5% normal gastrulæ, others abnormal morulæ	94
0	Controls	Motile blastulæ	0.4	Motile blastulæ	Gastrulæ	232

Experiments with Pure Light of λ2537A

To be certain that monochromatic light of λ2537A is the effective agent, a number of experiments were tried with pure light of this wavelength obtained from the monochromator already described. The unfertilized eggs were placed in a quartz cell and irradiated in the manner described in the previous paper (Giese and Leighton, 1935) usually for periods of 1, 4, 16, 64, 256 and 1,024 seconds. They were then transferred to watchglasses, inseminated and observed at hourly intervals until they had reached the 64-celled stage and then at 12-hour intervals until the gastrula stage. Seven series of experiments, the last three covering only the longer exposures, gave similar results and one series is plotted in Fig. 3.

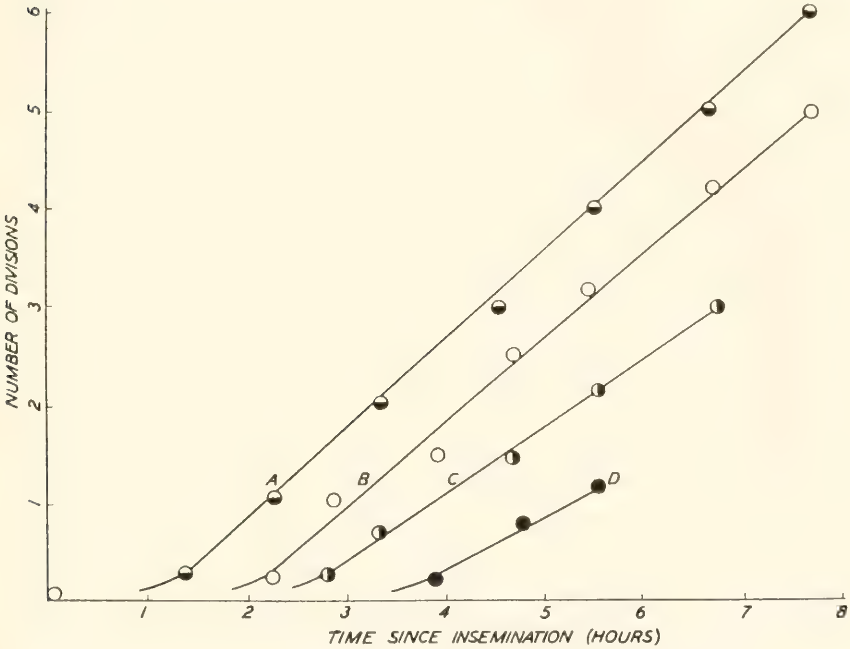

FIG. 3. Retardation of cleavage, λ 2537A; intensity, 9.74 ergs/mm.^{2}/sec.

A. Control.

B. Sixty-four seconds exposure, extinction per egg: 2.91 ergs or 3.74×10^{11} quanta.

C. Two hundred and fifty-six seconds exposure, extinction per egg: 11.67 ergs or 1.50×10^{12} quanta.

D. One thousand and twenty-four seconds exposure, extinction per egg: 46.69 ergs or 6.00×10^{12} quanta.

It will be noted that retardation is evident when the dosage is large enough. Smaller doses produced no noticeable retardation or acceleration. The results are in general similar to those already obtained with the mercury-argon discharge tube.

Extinction Measurements

It would be interesting to know the amount of energy which must be absorbed to produce the above effects. By interposing a cell first empty then full of eggs between the thermopile and the light source one can determine the fraction of the light incident upon the cell which is transmitted, I/I_0, where I is the intensity of the transmitted light and I_0 the intensity of the incident light. The fraction of the light extinguished, i.e., lost on passage through the cell can then be determined by subtracting the fraction transmitted from unity, $1 - I/I_0$. By determining the fraction of the area of the cell occupied by the eggs, A_e, one can determine the fraction of the light incident upon the eggs which is extinguished, $1 - I/I_0 \times 1/A_e$.

TABLE III

Data on extinction of light by sea urchin eggs

Exp.	No. eggs	A_e, fraction of area of cell bottom covered by eggs	$1 - I/I_0$ for λ's below:					$1 - I/I_0 \times \frac{1}{A_e}$ for λ's below:				
			2537A	4350A	5461A	5844A	6904A	2537A	4350A	5461A	5844A	6904A
1	3320	0.49	0.51	—	—	—	0.13	1.04	—	—	—	0.26
2	4431	0.65	0.63	0.38	0.27	0.23	0.14	0.97	0.58	0.42	0.35	0.22
3	3932	0.58	0.66	—	0.34	0.26	0.14	1.14	—	0.58	0.45	0.24
4	5964	0.88	—	—	—	0.38	0.25	—	—	—	0.43	0.28

A_e can be determined from the number of eggs, which can be counted, and the area of the egg effective in extinguishing light, which is approximately the area of a circle of the diameter of the egg. The average diameter of the egg used was the mean of the diameters of 50 eggs taken at random, $77.2 \pm 2.9\ \mu$ (two diameters were measured since almost one-half of the eggs were slightly ellipsoidal). A circle of this diameter has an area of $4{,}681\ \mu^2$. About 6,765 eggs would be necessary to completely cover the bottom of the cell used (area, $31.67 \times 10^6\ \mu^2$) with a layer one egg diameter thick. A_e is the number of eggs counted in a given experiment divided by 6,765.

The transmission of light of various wave-lengths by eggs was determined, the method used being similar in practically all respects to that described for *Paramecium* (Giese and Leighton, 1935), and the number of eggs was counted in each experiment. The experimental and derived data are recorded in Table III.

It is clear that practically all the light of λ2537A incident upon the eggs is extinguished, while at the longer wave-lengths a much smaller proportion is so lost. Part of the light extinguished is truly absorbed, part is lost by scattering from the surface of the egg and from the surfaces of small particles within the egg. Unfortunately it is very difficult to measure the scattering and it is impossible to obtain an approximation of the scattering as was done with *Paramecium* since unlike the latter the eggs absorb in the visible part of the spectrum as is quite obvious from the data in Table III. It is probable that as for *Paramecium* a considerable proportion of the light extinguished is actually scattered, possibly as much as 50 per cent of the total. However, the extinction measurements at least give the order of magnitude of the energy involved. The extinction by the eggs for the various experiments in Fig. 3 has been determined and the data have been added to that figure.

DISCUSSION

The data of Fig. 3 are interesting because they give an idea of the number of quanta which must be absorbed to produce an effect. Thus eggs extinguishing between 1.57×10^9 and 2.54×10^{10} quanta, and probably absorbing about half this quantity, were not visibly affected and cleaved comparably to controls, only after a dosage of about 3.74×10^{11} quanta per egg was the rate of cleavage definitely retarded. Only doses short of those producing cytolysis stop cleavage for eggs extinguishing 1.24×10^{13} quanta went on developing as far as the 8-celled stage in many cases. Were one to assume that the average molecule in the egg protoplasm had a mass of the order of magnitude of the mass of the egg-albumin molecule, one would find the egg to possess some 2.5×10^{12} molecules. The actual number is probably much larger, but the figure indicates that a fair proportion of the molecules are affected or that certain molecules have absorbed many quanta before an effect is evident. The data are also indicative of the high power of recovery from injury possessed by the egg protoplasm.

From the data presented one may conclude that for the wave-length and the dosage series used, which covers the range usually employed in similar experiments, there is no evidence of acceleration of cell division. Following large doses of radiation there is retardation; following smaller doses the rate of cleavage is not noticeably different from controls.

These results do not, however, exclude the possibility of a stimulative effect of doses of ultra-violet light much weaker than here employed. The radiations claimed by the Gurwitsch school of mitogenetic rays to be short ultra-violet and to be effective in increasing mitoses are postulated to be an entirely different order of magnitude from the radiations here used, in fact so weak as to defy physical detection. No attempt is here made to throw light upon this complex problem (see Rahn, 1936).

Summary

1. There is a threshold dosage between 10^{10} and 10^{11} quanta below which radiation of λ2537A produces no observable change in the rate of cleavage. Beyond this threshold the degree of retardation increases with the dose.

2. Many of the retarded eggs develop normally, but are slower in reaching a given stage; others continue developing for only a short time, the degree of differentiation reached being inversely proportional to the dosage.

3. Unfertilized eggs, eggs 15 and 90 minutes after insemination, and eggs in the first cleavage do not exhibit strikingly different susceptibilities to the rays, although the later stages appear to be somewhat more susceptible.

4. The quantity of radiant energy which the eggs can absorb before being affected is quite large, as indicated by the extinction measurements reported, and serves as a rough measure of the power of repair of the egg protoplasm.

5. A series of dosages from a dose which cytolyzes to one which has no retarding effect upon cleavage with light of λ2537A failed to induce artificial parthenogenesis.

6. No evidences were obtained over the dosage series investigated for acceleration of the rate of cleavage.

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THE HISTOLOGY OF THE RETRACTOR MUSCLES OF THYONE BRIAREUS LESUEUR

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The histology of invertebrate muscle has received relatively little investigation and there exist at the present time marked gaps in our knowledge of this subject. A more critical study of invertebrate muscle might possibly yield clues of interesting phylogenetic significance. Recent research in the field of holothurian muscle physiology has also made desirable a more complete histological study of these muscles.

The only extensive paper on the histology of holothurian muscle (Hall, 1927, on *Cucumaria*) completely misinterprets the essential structure and has been responsible for a number of erroneous conclusions by workers in muscle physiology.

This paper concerns itself with the more salient features of the histology of *Thyone* retractor muscle. The interpretations are opposed to the conclusions of Hall on the strikingly similar muscles of *Cucumaria*.

Materials and Methods

The retractor muscles of *Thyone* occur in five main bands which run from the lantern to points of attachment on the five longitudinal muscles of the body. The individual bands may be single or broken up into a number of divisions. They are extremely extensible and in adult specimens may vary in length from about 5 mm. in the contracted condition to 5 or 6 cm. in normal extension. Except for the two regions of attachment the muscles are free of the tissue and are easily removed.

The muscles were fixed in varying degrees of contraction in Bouin's or Helly's fixing fluids. In order to obtain normal extension it was necessary to immerse them for some time prior to fixation in 0.3 molar magnesium chloride solution. The muscles were dehydrated in alcohol, cleared in xylene, and imbedded in paraffin. Sections were cut at 5 and 10 micra.

Heidenhain's iron hæmatoxylin proved to be the most useful stain for the muscle fibers. Mallory's aniline blue and Dominici's stain were most favorable for the details of the connective tissue network.

Attempts were made to macerate the muscles in Bouin's fluid for periods up to three weeks and in varying strengths of nitric acid. These techniques, however, did not prove to be very satisfactory.

OBSERVATIONS

Microscopic examination of the fresh muscle reveals extremely long fibers grouped into indistinct bundles. In cross-sections of fixed and stained muscles this bundle arrangement is clearly discernible (Figs. 1 and 4). The inner portion of the muscle consists of hemicylindrical bundles containing from two to fifteen fibers which tend to be arranged about the periphery of each bundle. The outer portion of the muscle exhibits no definite bundles. Longitudinal sections reveal that the bundles do not maintain the same complement of fibers throughout their length. Fibers pass from one to the other of adjacent bundles and in the periphery of the muscle there is no definite association of fibers.

The spaces between the bundles and the fibers of the bundles are occupied by a delicate reticulum of connective tissue which is markedly condensed about each bundle (Figs. 1 and 4). The connective tissue appears to have the nature of a lattice-like framework with the thickest fibers running perpendicular to the muscle fibers. In sections of contracted muscle the elongated connective tissue nuclei are also seen to lie with their long axes in this direction. The connective tissue fibers are especially coarse and dense in the periphery of the muscle.

Connective tissue and tissue space constitute a relatively large proportion of the muscle in contrast to mammalian smooth muscle and some invertebrate muscle, e.g. retractor muscle of *Phascolosoma*. Steinbach (1937), in his studies on potassium and chloride in *Thyone* muscle, has estimated the volumes occupied by the muscle elements and tissue space. According to Steinbach, chloride diffuses freely into 43 per cent of the muscle—a figure which by his interpretation corresponds rather closely to the extrafibrillar space (tissue space and sarcoplasm). However, since Steinbach tentatively accepted the histological conclusions of Hall, his results may be reinterpreted. The structures which Steinbach, following Hall, interpreted as fibrils are actually fibers. Those structures interpreted as fibers are bundles of fibers. Thus it is found that chloride space corresponds closely to tissue space (47 per cent of *Thyone* muscle) as has commonly been believed (see Fenn, 1936), and does not include the sarcoplasm of the muscle fibers.

Fischer (1937) found that *Thyone* retractor muscle exhibited a birefringence of 60 per cent of that of *Phascolosoma* retractor muscle. (Histologically, *Phascolosoma* retractor muscle exhibits densely packed elements.) Since birefringence may be presumed to be associated with contractile elements, Fischer's figure suggests that these elements are fewer in number than in *Phascolosoma* muscle, and it may be further interpreted as lending support to the figure given above for tissue and chloride space in *Thyone* muscle.

As far as it has been possible to determine, the fibers are uninucleate. The nuclei occupy lateral positions on the fibers (Figs. 1 and 3), only rarely are they found within them (Fig. 4). In the contracted fiber the nuclei are rounded and are usually found to occupy a typical lateral position on the fiber. With the proper staining technique a layer of faintly staining cytoplasm may be seen to surround the nuclei. Often a thin strand of this cytoplasm forms the only connection between the nucleus and the muscle fiber. In the extended muscle the nuclei are elongated in the axes of the fibers and lie closely applied to them.

The appearance of the fibers in cross-section is somewhat variable. In the contracted condition they tend to be circular or semicircular (Fig. 1). Occasional fibers are polygonal. The stretched fibers may be circular or oval in transverse section, but are more often flattened or angular (Fig. 4). The fiber diameter varies from 5–10 μ in contraction to 2–4 μ in extension.

Teased preparations of muscle reveal the spindle-shaped character of the fibers. The tips of the fibers are long and tapering in extension (Fig. 5), bluntly rounded in contraction.

It was extremely difficult to determine the lengths of the muscle fibers since it was almost impossible to isolate these elements. The closely investing connective tissue binds the fibers securely together and all attempts at maceration and teasing were largely unsuccessful. Careful study of the best of these preparations, however, revealed that the fibers do not extend the whole length of the muscle. At

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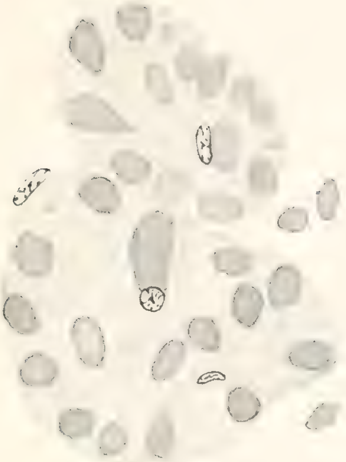
Explanation of Figures

All drawings were made with the aid of a camera lucida at a magnification of × 745.

FIG. 1. Cross-sections of two bundles of fibers from a contracted muscle.
FIG. 2. Portion of a longitudinal section of an extended muscle.
FIG. 3. Portion of a longitudinal section of a contracted muscle.
FIG. 4. Cross-sections of two bundles of fibers from an extended muscle.
FIG. 5. Fragment of a teased muscle.



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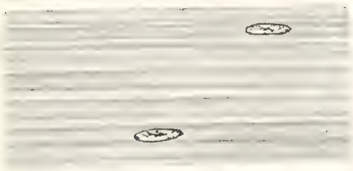

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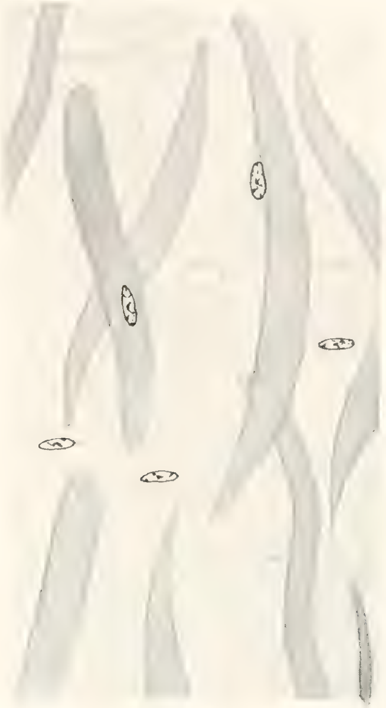

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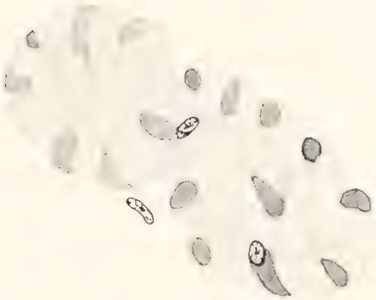

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Plate I

various points along a teased muscle natural unbroken tapering fiber terminations could be found (Fig. 5).

DISCUSSION

The contention of Hall (1927) that *Cucumaria* muscle consists of large fibers containing a few giant fibrils forms an untenable theory. From Hall's figures it is apparent that the histological picture of *Cucumaria* muscle is almost identical with that of *Thyone*. In *Thyone* muscle, however, the nucleated condition of the so-called fibrils is clearly evident. It must be concluded, therefore, that these are muscle fibers and not fibrils. It is true that the nuclei often have a tenuous attachment to the fibers, but this attachment is nevertheless definite, and, as previously stated, nuclei are occasionally found within the fibers. The free nuclei within the bundles, which Hall interpreted as muscle nuclei, are clearly constituents of the connective tissue. Further, the failure of the peripheral elements to be arranged into bundles (muscle fibers of Hall) does not support Hall's conclusion that these are fibrils.

The conception that the muscle fibers are long spindle-shaped elements is supported by the studies of Gerould (1896) on *Caudina* and Jordan (1914) on *Holothuria* and *Stichopus*. According to Gerould, muscle fiber nuclei in *Caudina* occupy a lateral position similar to those in *Thyone*.

du Buy (1936), studying the sensitivity of *Thyone* muscle to certain drugs, found that these muscles exhibited a physiological condition intermediate between vertebrate smooth and vertebrate striated muscle. Microscopically they resemble vertebrate smooth muscle. No histological similarity with vertebrate striated muscle can be detected with the possible exception of their extreme length. The fibers are homogeneous, devoid of striations or fibrillar structure.

SUMMARY

The retractor muscles of *Thyone* consist of long spindle-shaped smooth muscle fibers. The fibers appear to be uninucleate. The nuclei usually occupy a lateral position and are attached to the fiber by a delicate layer of cytoplasm. Occasional nuclei are found completely within the fibers.

In the center of the muscle the fibers occur in bundles of two to fifteen. No such organization is exhibited in the periphery of the muscle.

The fibers do not remain associated with a single bundle throughout their length but pass freely from one bundle to the other.

Measurements of fiber length were not obtained, but it is evident from study of teased preparations that they do not extend the full length of the muscle. Fiber diameter varies from 5–10 micra in contraction to 2–4 micra in extension.

The connective tissue forms a lattice-like network which is concentrated about each bundle and in the periphery of the muscle. The heaviest fibers of the reticulum tend to run perpendicular to the axes of the muscle fibers.

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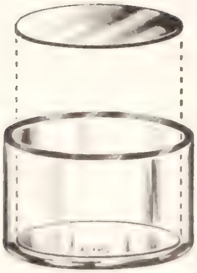

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THE

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A SIMPLE TELEOST KIDNEY IN THE GENUS CYCLOTHONE

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INTRODUCTION

Because it is the unit of structure and function in all vertebrate kidneys, the renal tubule contains the answer to many questions concerning the production of urine. Yet any theory which attempts to assign the various steps in this process to separate regions of the tubule must take into account the surprisingly wide degree of variation in tubule structure among the vertebrate groups. The relation of the presence or absence of the glomerulus to urine production and to the osmotic balance between the organism and different sorts of environment has been clearly outlined by H. W. Smith (1932) and others. The control of water content and salt concentration depends not only upon the action of the glomerulus, but also upon resorption and excretion by other tubule portions, such as the loop of Henle in mammals, and upon entirely extra-renal factors, such as the excretion of urea and salts by the gills of fishes.

The problem of further localizing kidney functions among the remaining segmental differentiations in the tubule proves to be more difficult. The fish kidney has been the subject of several cytological and histophysiological investigations contributing to this question, but confusion has resulted from the extreme diversity of kidney types in this group. Very few are found to contain all the segments of the typical vertebrate nephron, and Marshall (1934) has concluded that only the proximal convolute is common to all. There are as yet few clues to the benefits conferred by the other segments upon those fish which possess them. Among the forms which are able to adapt to sudden changes from fresh to salt water and back again, Grafflin (1937*a*) finds a lack of special development in any particular segment in relation to this remarkable ability, and concludes that it must be due to entirely extra-renal factors. Yet he does find indications of homologies in proximal convolute structure among those few which have been studied cytologically (see below). It is obvious that there is a need for observations on more forms, especially because of the wide diversity of tubule types in the few already examined. The present study was undertaken with this fact in mind, and it was thought that some of the little known deep sea fish might present interesting modifications. The inaccessibility of their habitat, and the numerous strange adaptations of body structure which they have produced there, might be taken as indications of an early arrival in the sea, which would have given plenty of time for kidney structure to reach the aglomerular condition considered most logical for marine forms. Actually, the few genera so far sectioned all show glomeruli. One of these, however, shows a strikingly simple type of kidney, and forms the subject of this paper.

TABLE I

Data on the seven Cyclothone sectioned for study of kidney in situ

Series Number	Length in mm.	Depth taken	Fixation	Serial sections	Portion of kidney included
1	55	—	Formalin and sea water	10 μ	Complete
2	40	—	Modified Held's	10 μ	Complete
3	56	0–2000 m.	Bouin's	5 μ	Complete
4	28	600 m.	Bouin's	5 μ	Complete
5	26	600 m.	Bouin's	5 μ	Complete
6	40	—	Modified Held's	10 μ	Posterior, from proximal convolute 2nd part to end.
7	45	—	Formalin and sea water	10 μ	Anterior, as far as proximal convolute 2nd part.

MATERIAL AND METHODS

The fish used in this investigation is a small deep sea form belonging to the genus *Cyclothone*. It is long and very slender, without eyes, and supplied with numerous minute photophores. The specimens range from 26 to 56 mm. in length, with corresponding diameters of 3 to 6 mm. respectively. They were obtained in the vicinity of Bermuda, mostly in open-net hauls at depths of 0 to 2,000 m. Some were caught in closing-net hauls at 600 meters and other depths. The collections were made from the ketch Atlantis of the Woods Hole Oceanographic Institution during the summers of 1936 and 1937, and specimens for the present work were obtained through the courtesy of Dr. J. H. Welsh of Harvard University.

The catch from each haul was usually preserved en masse in formalin and sea water. The extremely delicate body of the fish was shorn of much of the integument by crowding and rough treatment in the net, and was further twisted and distorted, and fixed in this state. Thus it was difficult to find a specimen in a straight condition suitable for sectioning. A few had been fixed separately in Bouin's or a modified Held's, but were in no better condition. None had been cut open, so that fixation was relatively poor. A group of those showing signs of better than average fixation plus a minimum of distortion was selected for a study of the kidney in situ by means of serial sections of the whole animal. In five of these, enough of the bodies were sectioned to include the entire urinary systems, which are reproduced approximately to scale (Figs. 1–5). A smaller portion of two others also was sectioned. All were stained with Harris hæmatoxylin and eosin. The data concerning the seven are given in Table I.

Observations

The *Cyclothone* kidney consists of a single pair of renal tubules extending parallel to the long axis of the body. They begin as two large, closely approximated Bowman's capsules, located dorsally at about the level of the last gill arches, and run a practically straight course side by side until they unite to form the bladder anterior to the anus. An investment of hemopoietic tissue surrounds the anterior half of the tubules, and just before entering the bladder they pass through a mass of glandular tissue which is probably the organ of Stannius.

Determination of the exact amount of regional differentiation in this nephron is difficult in the material available at present. There is a glomerulus, a neck segment, and a proximal convoluted segment of two portions. These four regions are marked off by histologically abrupt transitions. The second portion of the proximal convolute is followed by a thin intermediate segment, and this by a terminal segment somewhat like the distal convolute described in many tubules. These last two, however, are not set off by abrupt shifts in cell types. It is necessary to describe a long transitional region before the intermediate segment and, although the terminal segment appears rather suddenly, there is not a sharp histological transition from the intermediate segment.

Glomerulus and Neck

The capsule of Bowman is large, with a well-vascularized glomerular tuft which lacks the central avascular core observed in bird and reptile nephron by Vilter (1935). There is a large amount of intracapsular space, but it is not filled with coagulum, and does not resemble the degenerative enlargements or outpouchings seen by Grafflin (1929) in the goosefish capsule.

The two capsules are separated by a thin septum (Fig. 6) which becomes indistinct as it passes between the capillary tufts, so that these latter structures appear to form a single unit. It was not determined whether there was a separate blood supply for each side of the tuft.

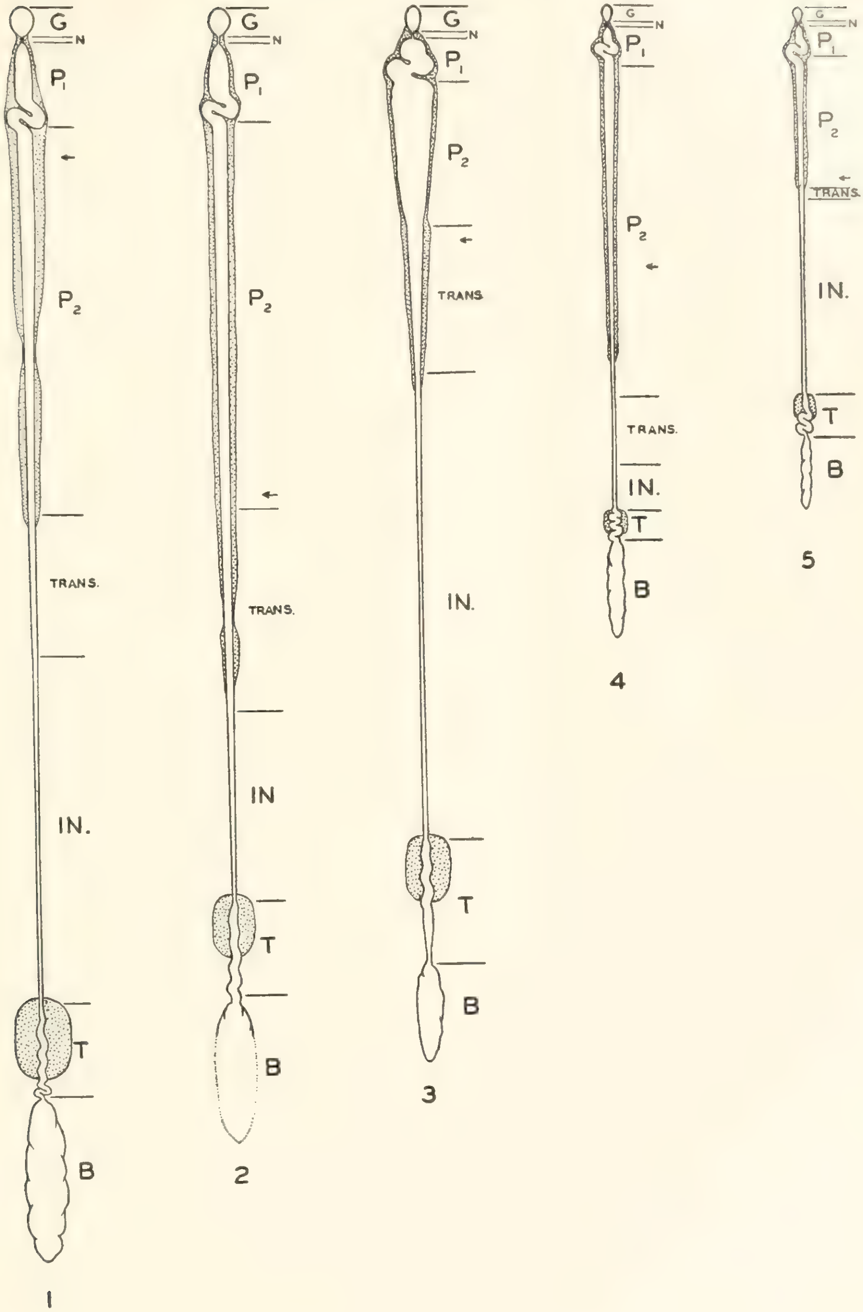
The short neck segments of the two tubules arise ventrolaterally from the glomerular capsules, and there is an almost immediate transition to the cells of the proximal convolute (Fig. 6). The neck cells bear cilia, but these are relatively few and very fine, requiring oil immersion for identification. The segment therefore does not give a picture comparable to the ciliated neck of other nephrons, such as that described by Guyton (1935) in the lungfish. The neck cells are of a low cuboidal type, 5 to 8 μ in height. Nuclei are small, irregular in shape, and stain densely. The cytoplasm also takes a hæmatoxylin stain. This is a short segment in all specimens, usually less than 100 μ. The diameter is small, but the lumen relatively large, so that there seems no question as to the functional nature of the glomerulus.

First Portion of Proximal Convolute

The cells of this region appear abruptly (Fig. 6). In the first few sections, the tubule wall shows a peculiar tufted structure due to the fact that the cell height varies from 12 to 50 μ (Figs. 7 and 8). Very soon, however, the cell height becomes comparatively constant (Fig. 9). Two of the specimens fail to show this irregularity.

In one member of the series (No. 7) the cells of the "tufted" region just described are filled with very large granules (Fig. 10) which crowd the nuclei against the basement membrane. These granules are spherical in shape and appear homogeneous in composition. Most of them stain a brilliant red with eosin, although a few remain colorless. Other specimens show the granules to a lesser extent, always smaller and fewer in number.

FIGS. 1 to 5. Diagrammatic reconstruction of kidneys of five *Cyclothone* specimens, × 10. Only one tubule is shown; the other joins it at beginning of bladder. Upper stippled areas, hemopoietic tissue; lower stippled areas, organ of Stannius. *G*, glomerulus; *N*, neck segment; P_1, first portion of proximal convolute; P_2, second portion of proximal convolute; *TRANS.*, transitional zone; *IN.*, intermediate segment; *T*, terminal segment; *B*, bladder. Arrows indicate level of appearance of first ciliated cells. Numbers correspond to those of the series described in Table I.


G
N
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1
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P_2
TRANS.
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B
5

FIGS. 1–5

The apical cytoplasm is always more or less granular, while that below the nucleus shows prominent striations, with fibrillæ arranged parallel to the long axis of the cell and closely packed together. The cytoplasm takes a deep eosin stain. There is a uniform brush border, rather coarsely fibrillar, and 5 to 8 μ in height.

Nuclei are oval, 4 to 5 μ in diameter, and heavily stained. Their position within the cell varies widely. In some specimens, they are located below the center of the cell in the first few sections, but later become central or even apical. In one series the nuclei are in the basal half of the cell throughout, while in another the position is always in the apical half (Figs. 7 and 8). The tubule enlarges greatly toward the end of this region, and convolutions appear. These always have the same simple structure, a double fold (Figs. 1 to 5).

Second Portion of Proximal Convolute

At this point there is an abrupt change (Figs. 11 and 12) to a cell type with lighter staining cytoplasm, larger nucleus, and high, irregular brush border. The cytoplasm shows very few granules, but is still striated, with fibrillæ loosely packed so that the cell appears less dense. The brush border becomes exceedingly irregular in form (Figs. 12 and 13) sometimes extending out into the lumen for a distance of 25 μ. Its structure is finely granular and sometimes vacuolated, without striations. Some portions have a droplet-like appearance as if about to be pinched off into the lumen, which suggests that these cells are engaged in some sort of apocrine secretion. In later sections, the border becomes lower and more regular, sometimes regaining a striated appearance.

The nuclei are larger than in the first portion and stain less heavily (Figs. 11 and 12). They are usually just above the center of the cell.

Fig. 6. Section of kidney at level of glomeruli, showing the close approximation of these bodies. Right tubule (above) sectioned at level of transition from neck (right) to P_1 (left). Other tubule (below) shows scattered P_1 cells among those of neck. Passage from glomerulus into neck is in an earlier section. × 180.

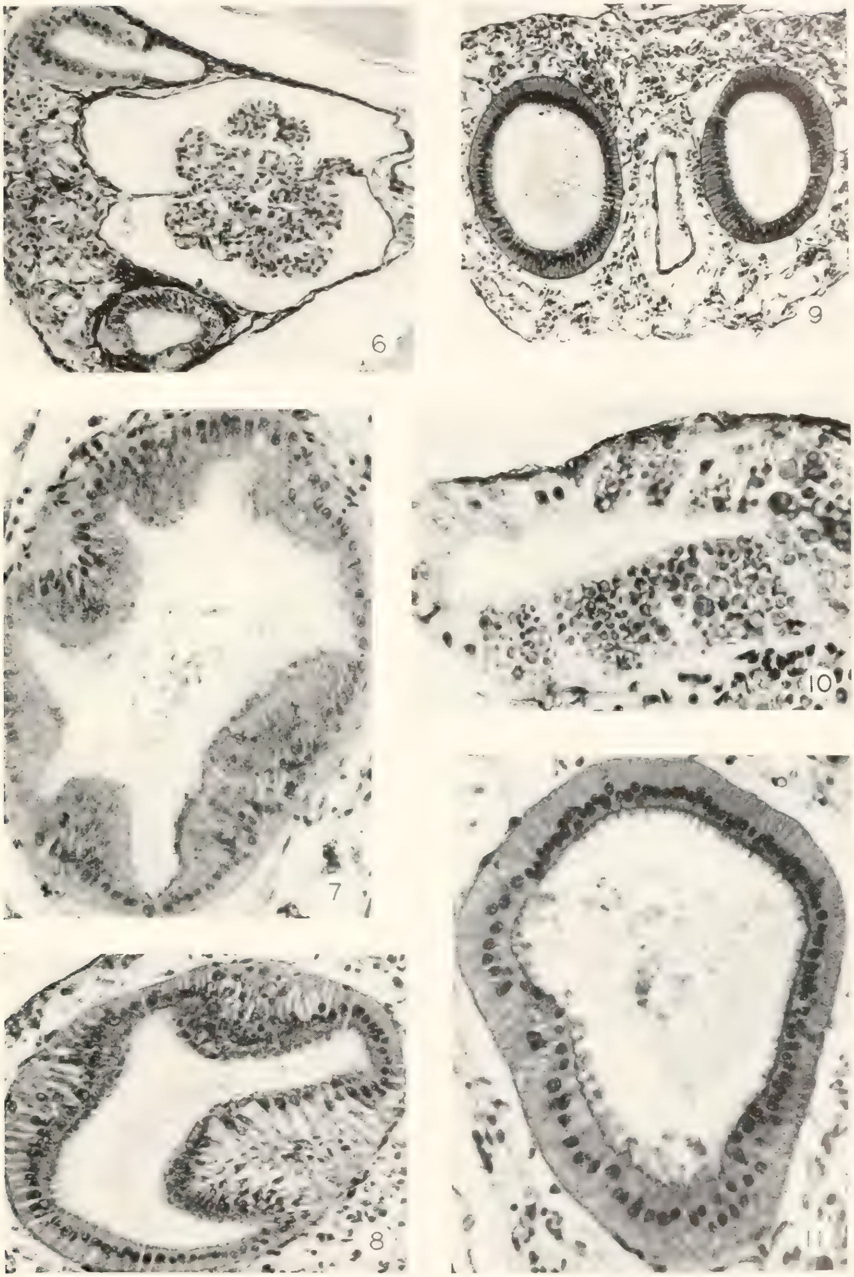
Fig. 7. Tufted region in early part of P_1; in this specimen the nuclei are basal and there is a large quantity of granular cytoplasm between them and the lumen. × 255.

Fig. 8. Same region of another specimen. Here the nuclei are apical, and there is much less of the granular cytoplasm. × 240.

Fig. 9. Central part of P_1. The tubule wall is even and the brush border regular. × 130

Fig. 10. Cells of P_1, showing remarkable number of large granules. These occur in only one specimen; see text. × 535.

Fig. 11. Section passing through region of transition from P_1 (cells of upper right half) to P_2 (lower left). × 280.



Figs. 6–11

Frequent double nuclei are seen, oriented in a line parallel to the vertical axis of the cell (Fig. 13).

The cells are wider than in the first portion, but only slightly higher, ranging from 15 to 20 μ in height near the transition from the first half of the segment. There is a steady decline in height of cell and brush border both, throughout this region.

Intermediate Segment

When the brush border is entirely gone and the cells are reduced to a cuboidal shape without prominent striations in the cytoplasm, a typical intermediate segment has been reached. This is separated from the proximal convolute by a long transitional zone in place of the abrupt change noted between the other segments. Two factors make it impossible to eliminate this zone. In the first place, the changes in cell height and nature of brush border take place gradually. The nucleus also slowly becomes smaller and more densely stained. In the second place, the cells on the medial side of each tubule retain the tall brush border, greater cell height, and fibrillar cytoplasm long after the cells of the rest of the epithelium are of the low cuboidal type of the intermediate segment, without brush border or cytoplasmic striations.

The tubule in the typical intermediate segment is very small and lies on the dorsal wall of the body cavity without any investment of hemopoietic tissue. It is usually less than 50 μ in diameter, and the cells are often much wider than high. The cytoplasm is homogeneous, dense, and rather heavily stained with eosin. Although the cell border is typically plain, there are frequently various sorts of bleb-like protrusions on the cell surfaces which are very much like a brush border.

FIG. 12. Same region under higher magnification. Note abruptness of transition; P cells are on right. × 630.

FIG. 13. Typical high brush border of P_2. It is highest on the medial wall of each tubule. × 240.

FIG. 14. Whole section; the kidney tubules lie just above the oesophagus (center). Dorsal side uppermost. The heart may be seen ventrally. × 9.

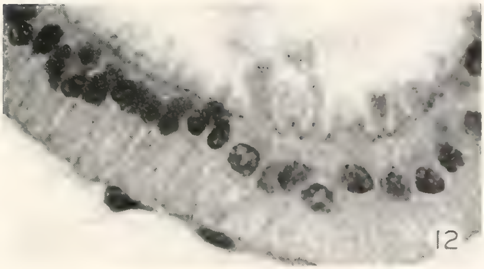
FIG. 15. Beginning of transitional zone. The epithelium is low, brush border is beginning to disappear (upper left) and ciliated cells are common. × 365.

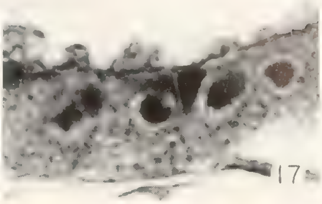
FIG. 16. Intermediate segment. Very low epithelium, no brush border, cilia numerous. Note small size of tubule. × 720.

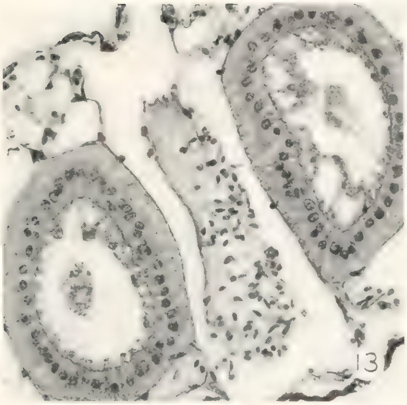
FIG. 17. Portion of tubule wall (P_2) to show a typical isolated ciliated cell. × 630.

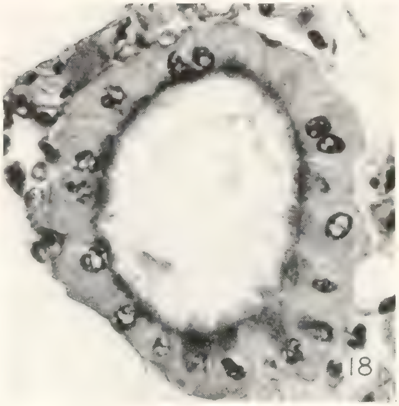
FIG. 18. Terminal segment. The strong suggestion of a brush border shown here is absent in most of the other specimens. × 645.

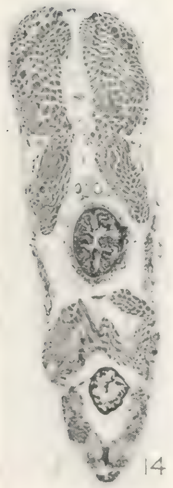
FIG. 19. Stannius organ, with kidney tubule at either end of glandular mass. × 90.

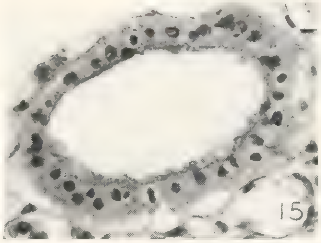


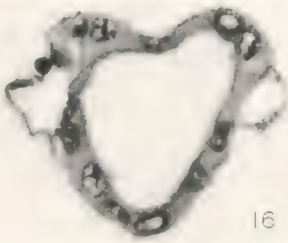


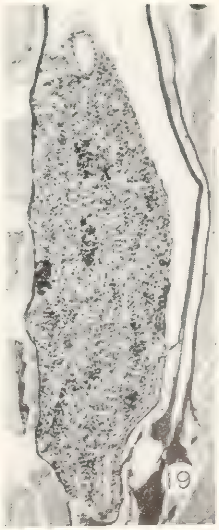












FIGS. 12–19

Cilia are fairly numerous, but are so extremely fine in texture that they never form a conspicuous feature of the tubule topography, and frequently require high power to be seen at all. Isolated ciliated cells first occur early in the second portion of the proximal convolute (Figs. 1 to 5). They are conspicuous as small cells without a brush border and with heavily stained nuclei, wedged between the other cells of the tubule wall (Fig. 17). The several cilia from each such cell are fused into one strand, only occasionally showing the individual elements. Rare at first, these cells become progressively more numerous up to the end of the intermediate segment, where there may be several in one cross-section (Fig. 15). In both structure and distribution they resemble the isolated ciliated cells in the second portion of the proximal convolute of the lungfish (Guyton, 1935).

Terminal Segment

As the tubule enters the organ of Stannius (Fig. 19) the cilia disappear, and there is a certain amount of restoration of the glandular type of epithelium. It shows an increase in thickness in all cases, usually about 20 per cent, but in two specimens the cells are doubled in height, and regain the striated cytoplasm. In one of these there is also a marked accumulation of basophilic granules in the apical region of the cell (Fig. 18). The cell margins are uneven and sometimes the roughness suggests a brush border (Fig. 18). In certain regions, however, the luminal border is smooth, while some of the nephrons show no sign of this surface modification at all, so that it is assumed this is not a brush border segment.

The transition to the terminal segment takes place within the distance covered by five or six sections, in one specimen, yet there is always a gradation of one cell type into the other, without an abrupt change.

It seems safe to conclude that there is no collecting segment, for the glandular epithelium frequently lasts unchanged to the point of union of the tubules at the bladder. Some tubules show a gradual return toward the structure of the intermediate segment, but the cells always remain somewhat taller.

Convolutions appear just before the start of the bladder. They are much less regular than the first set described above. The large specimens, which are females, show only a slight bending of the tubule, while in the small ones (Figs. 4 and 5) it may go through several tortuous loops. It is interesting to note that these smaller individuals are males, a fact which suggests that the extra convolutions might bear some relation to the "sexual segment" observed in certain vertebrate male kidney tubules. However, no connection was observed between the testis and the kidney in either of the two males sectioned.

In the midst of these convolutions, the tubule leaves the organ of Stannius and descends ventrally to the bladder. This organ is lined with a low cuboidal epithelium, in which the curved tops of the cells give a characteristic biscuit-like appearance. A very thin coat of smooth muscle completes the structure. An external opening is found behind that of the digestive tube, and close to that of the reproductive tract, at least in the case of the female. Neither of the male specimens shows an open duct leading from the testis, although a very thin strand of tissue can be traced back from this organ toward the region of the anus.

Discussion

All the *Cyclothone* kidneys so far sectioned show a high degree of similarity between corresponding parts of the tubules from the glomerulus on through most of the second portion of the proximal convolute. Beyond this region differences are encountered in the nature of the epithelium, making it difficult to find characters common to all, and next to impossible to draw comparisons with other tubule types.

The confusion with regard to structure in this region is not entirely due to an unusual tendency toward individual variations. The proximal half of the tubule shows decidedly better fixation of important cellular features, probably because penetration was much faster there in the region of the gills than it was posteriorly where the kidney is surrounded by the thick muscle layers of the body wall (Fig. 19).

Audigé (1910) states that the typical teleost kidney may consist of three parts, the head, middle, and hind kidney. The head kidney in young fish contains two large glomeruli derived from the pronephros. These are rarely seen in adults, although Price (1910) describes one in *Bdellostoma stouti.* In *Cyclothone* there is a lack of any opening into the cœlom such as would be expected with a pronephric tubule, and in addition the glomerulus is not external. Furthermore, there is a direct passage toward a urinary bladder along a tubule whose epithelium is characteristic of the renal tubules of typical vertebrate mesonephroi. Nevertheless, the extreme anterior location of these two bodies suggests that they may be modified pronephric glomeruli, associated posteriorly with a pronephric duct which has undergone modification into a typical secretory tubule.

There is a remarkable variation in tubule sizes among the seven individuals sectioned. In the typical vertebrate kidney all tubules are identical, but their number varies with the size of the kidney and of the animal containing it. Nash (1931) has pointed out the constancy of the ratio between tubule number and body surface in several fish. In the present example, however, the tubule number is constant. The amount of kidney tissue is nevertheless adjusted to the size of the animal, simply by a change in size and length of the tubules themselves. The size of the fish from which these tubules are taken is given in Table I.

Even the smallest of the five illustrated here is a rather large tubule in comparison with those of other fish. Table II lists the approximate measurements of the largest and smallest tubules of the *Cyclothone* series (Figs. 1 and 5). These may be compared with tubule sizes reported by Nash (1931) for a large number of species. Tubule lengths he found usually between 1 and 9 mm.; diameters ranged upwards from 25 μ. Glomeruli were commonly between 35 and 95 μ. But isolated examples in his list far exceed this range: the hagfish glomerulus is 500 μ in diameter, and the nephron of the skate attains a length of 19 mm. Therefore the *Cyclothone* nephron comes well within the limits observed by Nash. In only one specimen is the tubule diameter really exceptional; the proximal convolute of No. 3 measures 500 μ in several places.

TABLE II

Approximate measures of largest and smallest tubules of Cyclothone series

	Segment diameters (μ)				Total length
	Glomerulus	Prox. conv.	Interm.	Terminal	
Largest (No. 1).............	250	200	50	60	14.2 mm.
Smallest (No. 5)............	130	100	30	50	5.6 mm.

The lack of special cytological preparations makes it difficult to homologize the finer structure of the different tubule regions with those of other kidneys. In his description of the cell types in the two portions of the proximal convolute of the sculpin, Grafflin (1937*c*) lists, among others, these three points of difference: the second part shows smaller nuclei, a more eosinophilic cytoplasm, and lower brush border, than the first. This is exactly the reverse of the condition as regards these three characters in the *Cyclothone* proximal convolute divisions. This suggests that the two portions of the proximal convolute in these two animals should be homologized in reverse order. Yet throughout the whole proximal convolute of the *Cyclothone* nephron, the cells show a greater density of the cytoplasm on the luminal side of the nucleus, which is just the opposite of the condition reported in the sculpin.

In attempting to set up a general picture of homologies among the different fish kidneys, Grafflin (1937*d*) concludes that the cell type of the second half of the proximal convolute of the sculpin is homologous with the same region in the tubule of the eel, and with the entire proximal convolute of the toadfish which shows no signs of division into two parts. Also, the first portion of the proximal convolute in eel and sculpin presents a second homology. Returning to *Cyclothone,* if we try to make a comparison between the first part of the proximal convolute, and the second part of that segment in the eel, as we tried to do with the sculpin above, we find again the same similarity as to eosinophilic cytoplasm and lower brush border. The difference in nuclear size, however, is not mentioned in Grafflin's description of the eel nephron (1937*b*), and our comparison is further weakened by the appearance of his figure showing the point of transition between the two cell types. This photograph shows a much larger cell in the second portion of the tubule and suggests that the homology with *Cyclothone* may well be a direct one, part one with part one and part two with part two. This view is further strengthened by the fact that a " zone of granular cells " sometimes appears in the first half of the proximal convolute of the eel, just as does a similar zone in one of the *Cyclothone* specimens (see above and Fig. 10).

The nephron of the lungfish, *Lepidosiren paradoxa,* according to the description of Guyton (1935) is quite similar in size to that of the larger cyclothones, and except for having an initial collecting segment, it is divided into the same general regions. Grafflin finds that this tubule does not fit very well into his scheme of homologies among proximal convolutes. The first portion of that segment in the lungfish resembles the second in sculpin and eel, while the second part doesn't resemble anything at all in the other two. The principal cell in this second part has an inconspicuous brush border and lacks prominent vertical striations in the cytoplasm. While these characters make it as foreign to *Cyclothone* as to the eel and sculpin, it is nevertheless worth noting that it shows a denser apical cytoplasm much like the cells of the proximal convolute in the *Cyclothone* nephron. The shape and distribution (increasing toward the distal end) of the ciliated cells in the second part of that segment is similar in these two fish also.

It should be emphasized that the problem of homologies between cell types of the various segments in different tubules depends upon the constancy of the characters chosen for comparison. In the sculpin, the nuclei of the cells of the first part of the proximal convolute are described as being usually in a central position, although sometimes at the base of the cell. In the same region of the eel, nuclear position is stated as almost invariably basal. In the *Cyclothone* tubules, it is possible to compare areas at exactly the same distance along each segment. When this is done one finds, using the first part of the proximal convolute as an example, a variation all the way from basal nuclei to apical ones (Figs. 7 and 8). The coarsely granular cytoplasm in the apical region of cells in both these tubules suggests that this area is engaged in active secretion, and that the nuclear position may simply vary with changes in the cytoplasmic volume at the apical end. That such changes may follow a definite cycle can be seen from the histophysiological studies made by von Möllendorff (1937) on fish kidneys. He observes a secretory cycle involving changes in both cell border and cytoplasmic content. Such a series of repeated changes make it risky to consider many cytological features specific for one tubule region, until it be determined whether they are subject to change under various physiological states of the segment in question.

In conclusion I wish to state my sincere appreciation for the kindness of Professor A. B. Dawson of Harvard University in suggesting this problem and in offering valuable advice at all times in the course of the work.

SUMMARY

The *Cyclothone* kidney is extremely simple. The entire system consists of two tubules running a practically straight course side by side and uniting at the bladder. They are surrounded anteriorly by hemopoietic tissue and posteriorly by the organ of Stannius.

The tubules can be divided into the following histologically distinct portions: glomerular capsule; neck segment; proximal convolute, with two types of brush border epithelium; ciliated intermediate segment; and terminal segment. An abrupt change in cell type separates the neck segment and the two parts of the proximal convolute, but the intermediate segment arises after a long zone of slow transition, and the cells of the terminal segment also appear gradually.

The cell types of the different regions of the nephron are described, with a discussion of the problems of homologies between tubule segments of different kidneys.

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EYES OF DEEP-SEA CRUSTACEANS

II. Sergestidae

J. H. WELSH AND F. A. CHACE, JR.

(From the Woods Hole Oceanographic Institution[1] *and the Biological Laboratories, Harvard University)*

Introduction

In the first of this series of papers which dealt with the eyes of the Acanthephyridae (Welsh and Chace, 1937) it was shown that *Hymenodora glacialis,* a species of deep-sea prawn which normally lives at a level below the photic zone, has quite degenerate eyes, while related species which normally inhabit the photic zone have well-developed and obviously functional eyes. It was also shown that among the acanthephyrids of the photic zone those possessing photophores have larger eyes in proportion to body size than those which lack the means to produce light of their own. Since such correlations between the structural development of the eye, light intensity and the ability to produce light are of considerable interest from the standpoint of adaptation, the present study of the eyes of the Sergestidae was undertaken with these points in mind.

Much of the literature dealing with the eyes of deep-sea crustaceans was cited in the paper already referred to and since collecting methods were also discussed in this paper and one by Welsh, Chace and Nunnemacher (1937), it will be unnecessary to discuss these matters further.

We are greatly indebted to Dr. H. B. Bigelow for the privilege of continuing this work and to the Milton Fund Committee of Harvard University for a grant which cared for certain of the expenses of the investigation.

Characteristics and Distribution of the Sergestidae

The prawns which make up the family Sergestidae form a well-defined, aberrant group of the most primitive tribe of the Natantia, the Penaeidea. Since the species dealt with in this paper belong, in the main, to the genus *Sergestes* it is not necessary to dwell on the four remaining genera of the group.

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[1] Contribution No. 176.

All of the species of *Sergestes* are slender-bodied animals with long, slender appendages (Fig. 1) and an integument that is never as firm as in most of the acanthephyrids. Despite their fragile appearance, most of the species retain their form and appendages fully as well as most other pelagic decapods when subjected to the usual handling. The last two pairs of thoracic legs are flattened and shorter than the other three pairs and fringed with long hairs. These, in conjunction with the long, well-developed pleopods suggest that the sergestids are among the fastest swimming of the pelagic Crustacea. Further indication that such is the case is provided by the fact that very few adult specimens and practically none of the mature individuals of the larger species were captured until the advent of the motor-driven vessel which was able to tow nets at a fairly rapid rate of speed. In collections now made with modern ships and apparatus, however, sergestids are very frequently encountered, and a number of the North Atlantic species are apparently reasonably abundant in the midwater area; possibly the commonest species in any one region is the luminous species, *Sergestes lucens* Hansen, from off Japan, of which 10 million pounds are said to be taken annually by commercial fisheries.

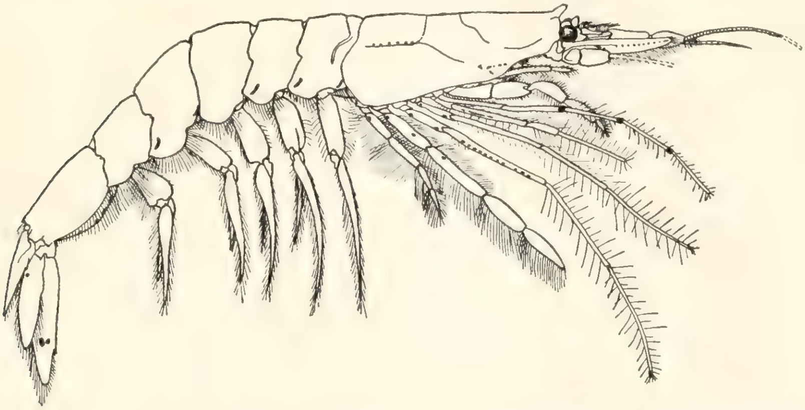

FIG. 1. *Sergestes grandis* Sund, female, showing certain of the features which characterize sergestids. Photophores, which are present only in certain species, are indicated by solid black spots.

The lack of adult individuals and the extremely long and complicated larval history of these animals were the chief obstacles in the path of the correct determination of species by the early carcinologists. Innumerable species have been described, a great many on larval forms, and it was not until the last few years that any idea of the number of valid species could be learned. It is estimated that there are between thirty and thirty-five species of *Sergestes,* and even now several comparisons, particularly between Atlantic and Pacific specimens, are necessary before the exact number of known forms can be ascertained.

Although they inhabit the general "black fish-red prawn" area, only two of the sixteen species known from the North Atlantic are distinctly red in color. The others range from a deep rose pink and a pattern of red spots to almost perfectly transparent forms in which the internal organs may be clearly seen.

In 1903 photophores or luminescent organs were observed for the first time in any species of the genus and since then they have been described in four other species. Recently organs which are probably photophores have been detected in three other Atlantic species. It is very probable that further study of fresh material will show that about a dozen species possess these structures. So far as we are aware no instance of the discharge of a "luminous cloud" such as observed in several other bathypelagic forms has as yet been recorded for these forms.

The species are most abundant in tropical and sub-tropical areas, being most numerous in the Atlantic between the Tropic of Capricorn and 30° North Latitude. Although fourteen of the sixteen species known from the North Atlantic area were taken by "Atlantis" at Stations 2666 (39° N., 70° W.) and 2667 (35° N., 69° 36′ W.), only three species have been taken by any expeditions north of 47° N. Lat. The horizontal distribution of the species of *Sergestes* is similar to that of other bathypelagic decapods; several species have been taken at numerous localities throughout the world, while others have a restricted distribution with closely related species inhabiting corresponding areas in other oceans.

Comparatively little is known of the vertical distribution of these animals. This is far from surprising when one realizes that previous expeditions have for the most part used open nets, which are perhaps more likely to catch animals when the net is being raised to the surface than at any other time. Also, most expeditions have covered a wide area and an attempt has been made in the reports to combine into one table the depth records for each species from these widely scattered localities. When one considers that probably no condition save pressure is everywhere constant at a given depth; when one realizes that light intensity, temperature, oxygen content and the chemical composition of the water all vary markedly at different localities at a stated depth, it is no less than amazing that certain species are apparently found at so nearly similar depths in all parts of the area in which they are encountered. Until exhaustive collections are made with closing nets at single stations and the results examined separately we will continue to be quite ignorant of the optimum conditions preferred by the various species. Table I gives a very rough idea of the depths at which the various species may be encountered in the area explored by "Atlantis." The records for *S. arcticus* were made at Stations 2463 and 2894, and for *S. mollis* at Stations 2475, 2666, 2667 and 2894; the other "Atlantis" specimens were all taken at Station 2667. Since most of the tows at Station 2667, from which most of the material was obtained, were made only at 400 and 800 meters, very little is known of the lower limits of any of these species. It is hoped that work on "Atlantis" in the near future at Station 2667 in greater depths will make the picture more nearly complete.

TABLE I

Depths in meters at which adult specimens of *Sergestes* have been taken with closing nets by "*Atlantis.*" [2]

	0	200	400	600	800	1000	1200	1400	1600	1800	2000	2050
Sergestes												
mollis[3]	..	..	..	X	X	X	..	..	X	X	X	X
sargassi	..	..	X	..	X	..	..	..	X			
pectinatus	..	X	X	X	X							
arcticus	..	X	X	X	X	X						
corniculum	..	..	X	..	X							
edwardsi	..	..	..	..	X							
cornutus	X	..	X									
vigilax	X	..	X	..	X	X						
atlanticus	X	..	X	..	X							
tenuiremis	..	..	..	..	X							
grandis	..	..	X	..	X							
crassus	..	..	X	..	X							
robustus	..	X	X	X	X							

EXTERNAL FEATURES OF THE EYES

The accompanying figures (2–9) and Table II illustrate the amount of variation in the eyes of *Sergestes.* Since the body is less compressed and the bases of the antennal scales are broader in these forms than in the acanthephyrids, the eyes are borne on relatively longer stalks to enable the animal to see in all directions when the eyes are extended laterally. Since the length of the carapace is apparently not a constant fraction of the total body length and since such robust species as *S. crassus* have a much shorter carapace in proportion to body size than slender species like *S. corniculum,* it was found that the ratio of the diameter of the cornea to the carapace length showed less clearly the actual comparative sizes of the eyes than did the ratio of the diameter of the cornea to the entire length of the cornea and eyestalk and the portion of the entire cornea and stalk occupied by the cornea. Even the latter comparisons are far from ideal as some species such as *S. arcticus* apparently have a longer stalk than others, but a more trustworthy comparison could not be found. Every part of a prawn which might be compared to the diameter of the eye is found to show some specific variation.

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[2] The records of surface catches of adults of *S. atlanticus*, *S. cornutus* and *S. vigilax* have been taken from the reports of other expeditions.

[3] The catches of *S. mollis* at 600, 800 and 1,000 meters were all made at Station 2894. The lower transparency of the water in this region as compared with the Sargasso Sea, where most of the other hauls were made, may account, in part, for this occurrence of *S. mollis* at depths so much shallower than those at which it is usually taken.

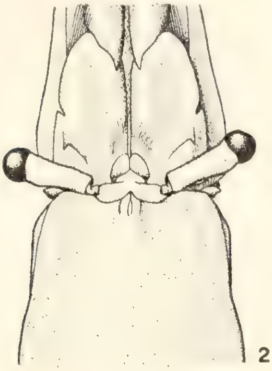
TABLE II

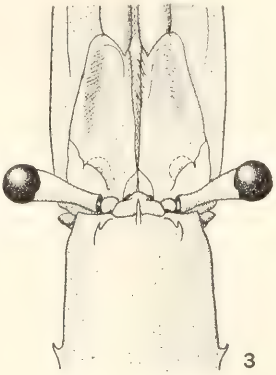
Relative size of the cornea as compared with the length of the carapace and the combined length of eyestalk and cornea in thirteen species of *Sergestes.*

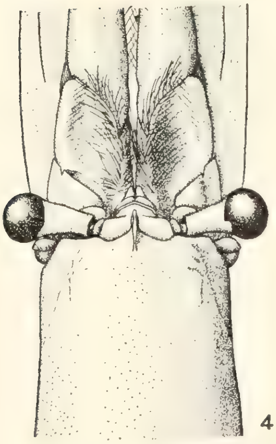
Species	Ratio Diameter of cornea to Length of cornea plus eyestalk	Ratio Length of cornea to Length of cornea plus eyestalk	Ratio Diameter of cornea to Length of carapace
Sergestes			
mollis	0.34	0.25	0.052
sargassi	0.40	0.31	0.085
pectinatus	0.45	0.36	0.099
arcticus	0.46	0.38	0.090
corniculum	0.47	0.43	0.072
edwardsi	0.54	0.37	0.098
cornutus	0.56	0.34	0.092
vigilax	0.57	0.44	0.097
atlanticus	0.58	0.42	0.105
tenuiremis*	0.63	0.53	0.088
grandis*	0.63	0.57	0.081
crassus*	0.65	0.50	0.132
robustus*	0.67	0.67	0.115

* Luminescent species.

It is apparent from the accompanying figures that by far the smallest eyes in proportion to body size are to be found in *S. mollis* (Fig. 2), the only Atlantic species that obviously is restricted, for the most part, below the 1,000 meter zone. This species is a soft-bodied form, red in color, and it recalls to mind *Hymenodora* among the acanthephyrids, although the eyes are far less degenerate than in the latter. One other sergestid, *Petalidium obesum,* very probably inhabits a similar depth and the eyes in it are possibly even smaller than in *S. mollis.* The form with the largest eyes, *S. robustus* (Fig. 5) is not, as might be expected, one of the species that frequents the surface layers. In fact, those four species which have the broadest eyes in proportion to the stalk, *S.*







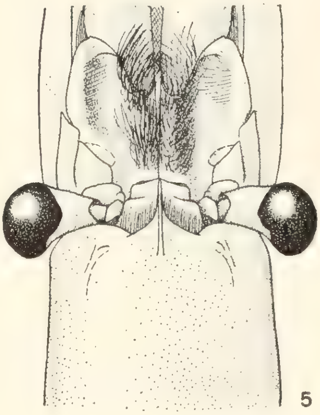


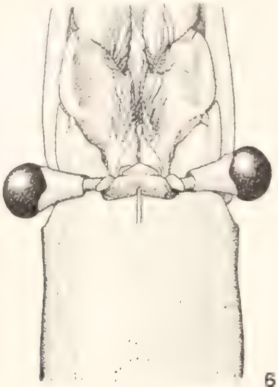
Fig. 2. *Sergestes mollis* Smith, male, 48 mm. in length. A species which is usually taken below the photic zone. Photophores absent. × 5.

Fig. 3. *Sergestes arcticus* Kröyer, male, 40 mm. in length. Photophores absent. × 5.

Fig. 4. *Sergestes grandis* Sund, female, 56 mm. in length. Photophores present. × 5.

Fig. 5. *Sergestes robustus* Smith, male, 58 mm. in length. Photophores present. × 5.

tenuiremis (Fig. 6), *S. grandis* (Fig. 4), *S. crassus* (Fig. 8), and *S. robustus,* are seldom taken in the upper 400 meters. Next in order above these (possibly by chance) are the three species which have been found at the surface, and between this group and *S. mollis* are five species with rather small eyes which, so far as is known, frequent the lower part of the photic zone.[4]



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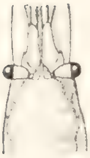

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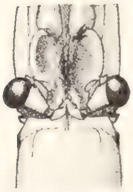

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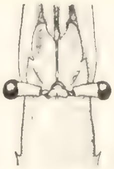

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FIG. 6. *Sergestes tenuiremis* Kröyer, male, 52 mm. in length. Photophores present. × 5.

FIG. 7. *Sergestes cornutus* Kröyer, female, 14 mm. in length. Photophores absent. × 5.

FIG. 8. *Sergestes crassus* Hansen, male, 24 mm. in length. Photophores present. × 5.

FIG. 9. *Sergestes atlanticus* H. Milne Edwards, female, 18 mm. in length. Photophores absent. × 5.

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[4] The investigations of Welsh, Chace and Nunnemacher (1937), and more recent unpublished studies, indicate that certain of the sergestids undergo extensive diurnal vertical migrations. The level of maximum numbers of a given species may be between the surface and 200 meters during the night and between 600 and 800 meters during the day. Therefore it is impossible to state that a certain species lives normally at a certain depth, and the most one can say is that the majority of the sergestids live in the photic zone and perhaps only one species in the North Atlantic, *S. mollis,* normally occurs below the level to which light penetrates.

If these figures present a true picture of the relative sizes of the eyes in the various species examined, one would naturally look for some factor to account for the large eyes of the four species at the bottom of the table. We believe that the answer may be found in the possibility that those species possess luminescent organs or photophores.

Until the sergestids of the "Michael Sars" expedition were reported upon by Sund in 1920 there was but one North Atlantic species, *S. splendens* Hansen, which was known to have photophores. Sund mentioned some pigmented spots in three other species, *S. grandis, S. crassus* (=*S. splendens* Sund), and *S. robustus,* which he suggested might be luminous organs. From the material of these three species collected by "Atlantis" we have been able to confirm his observations, and, although no histological preparations have as yet been made of this material, the position of the spots parallels so closely that of the photophores in those species which unquestionably possess them that there seems little doubt that such is their function. *S. crassus* and *S. robustus* apparently have these spots only on the antennal scales and outer uropods, while *S. grandis* (Fig. 1) has them scattered about the body and legs, particularly under the thorax and abdomen. In one large specimen of the latter species no less than 167 of these structures were counted. Hansen (1922) apparently doubted that these spots were actually photophores since they were not equipped with the lens-like structure found in the photophores of most other decapods and so become invisible in specimens preserved in alcohol or for too long in formalin. However, Kemp (1925) has described what he believes to be luminous organs in three species of pandalids from the Indian Ocean and these differ in structure from those which are known of other decapods in lacking any external trace of a lens.

If, then, the assumption is allowed that these structures are luminescent, only *S. tenuiremis* remains of those species which have the ratio of cornea to length of eyestalk greater than 0.60 and the ratio of the length of the cornea to the eyestalk greater than 0.50. Because no luminiscent structures were known in that species, a careful examination of a large male specimen was made. Although the specimen had been in formalin for about seven months and the characteristic red color had consequently disappeared, a pair of large, whitish organs were found in the coxae of the last pair of thoracic legs near the openings of the vasa deferentia. Sections made from one of these organs proved that they were almost certainly a cluster of at least three large photophores with well developed lenses entirely enveloped by the surrounding tissues. Their position and the absence of an external lens closely parallels the structures described by Kemp in the Indian pandalids.

The evidence presented would indicate that there is some correlation between the size of the cornea in the species of *Sergestes* which live near the limit of light penetration and the presence or absence of luminescent organs. In the other five species which are known to have photophores, *S. challengeri* Hansen, *S. fulgens* Hansen, *S. prehensilis* Bate and *S. splendens* Hansen the eyes are described and figured as large, although the figures are usually not sufficiently accurate to permit actual measurements. O. Pesta (1918) described structures on the inner side of the carapace in *S. corniculum* which he suggested might be photophores, but if these spots do prove to be luminous the proposed theory obviously does not hold for this species.[5]

Approximately one half of the described North Atlantic species possess a small tubercle on the inner margin of the eyestalk near the cornea. It may be of interest to list here the species which have or do not have this tubercle, although there seems to be no correlation between the presence or absence of such a structure and the presence or absence of photophores or the size of the cornea.

Tubercle present	Tubercle absent
S. armatus	*S. arcticus*
S. corniculum	*S. atlanticus*
S. crassus	*S. cornutus*
S. grandis	*S. edwardsi*
S. pectinatus	*S. mollis*
S. sargassi	*S. robustus*
S. tenuiremis	*S. splendens*
	S. vigilax

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[5] According to Burkenroad (1937) Organs of Pesta are probably present in all species of sergestids excepting *S. mollis, S. tenuiremis* and *S. challengeri.*

Figures 10–15 inclusive are all from dorso-ventral sections of eyes, unstained, × 32.

FIG. 10. Photomicrograph of a section of an eye of *Sergestes corniculum* made by means of transmitted light.

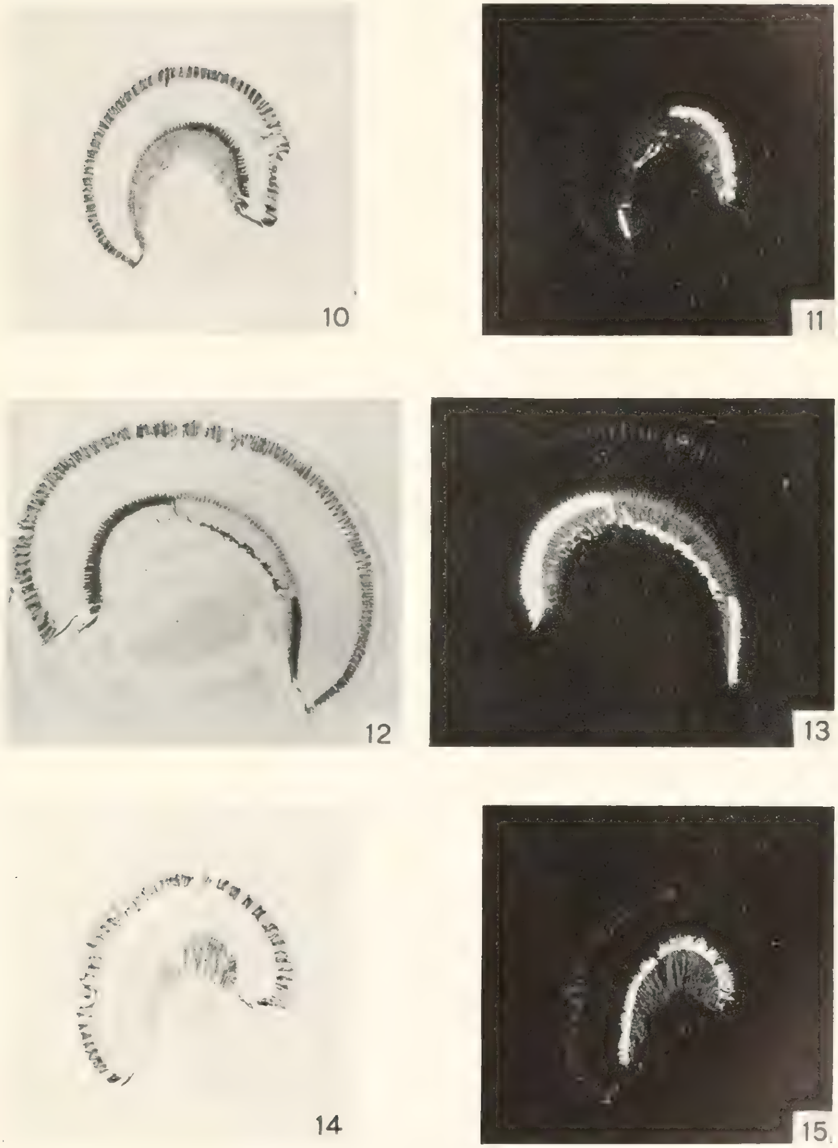
FIG. 11. The same preparation as in Fig. 10 photographed by means of dark-field illumination. The reflecting pigment which is more abundant in the dorsal part of the eye may be readily distinguished from the screening pigment.

FIG. 12. Photomicrograph of a section of an eye of *Sergestes robustus* made by means of transmitted light.

FIG. 13. The same preparation as in Fig. 12 photographed by means of dark-field illumination. The reflecting pigment is more abundant in the dorsal region which is toward the left.

FIG. 14. Photomicrograph of a section of an eye of *Sergestes mollis* made by means of transmitted light.

FIG. 15. The same preparation as in Fig. 14 photographed by means of dark-field illumination. The reflecting pigment is more evenly distributed throughout the region of the eye where it normally occurs.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11
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Figs. 10–15

Structure and Pigmentation of the Eyes

The eyes of eight species of sergestids were sectioned and examined histologically. The structure of these eyes was found to be essentially like that of deep and shallow-water shrimps and prawns whose eyes have been frequently described. One feature common to all was found to be the large and clearly defined rhabdomes, the receptor elements of the eye. The most striking differences were seen in the amounts and distribution of screening and reflecting pigments.

The eyes of *S. grandis* and *S. tenuiremis* resemble those of *S. robustus* (Figs. 12 and 13). It has already been pointed out that these three species possess photophores and this probably accounts for their large eyes. This may also be the reason for the large amount of reflecting pigment found in these eyes. Its distribution is such that the periphery of the eye may be more sensitive to differences in light intensity than the central portion, and the larger amount of proximal pigment in the central portion of the eye may conceivably increase the visual acuity of the region by a partial isolation of the rhabdomes from one another.

S. corniculum (Figs. 10 and 11), *S. arcticus*, *S. armatus* and *Petalidium obesum* probably do not possess photophores and certain of them may inhabit a region of higher light intensity than the three mentioned above. Of these forms only the first has any reflecting pigment in the eye, the others completely lacking this set of pigment. *S. arcticus* and *P. obesum* have a large amount of black, proximal, screening pigment.

Since *S. mollis* normally lives below the photic zone, as does the acanthephyrid *Hymenodora glacialis*, one might expect to find that its eyes were equally degenerate but such is not the case. They are the smallest, in relation to body size, of all the sergestids examined by us, but structurally the eye shows very little modification which may be correlated with life in a zone to which sunlight does not penetrate (Figs. 14 and 15). The even distribution of reflecting pigment may conceivably be related to the conditions under which the animal lives, but there are fairly large amounts of distal and proximal screening pigments. In *Hymenodora* (Welsh and Chace, 1937) it was shown that screening pigments were completely lacking, the rhabdomes had disappeared and there was an abnormally large amount of reflecting pigment. Hence the eye was considered to be quite degenerate and capable of doing no more than registering changes in light intensity.

Decapod crustaceans which are known with certainty to dwell exclusively in the vast intermediate region of the sea, between the lower limit of the photic zone and the bottom (excepting the bottom fauna), are not numerous as regards species. Until more have been studied it will be impossible to determine the exact trend in the degeneration of the eye as a result of living in complete darkness. From the work thus far carried out, however, it appears that depth, hence light intensity, modifies the eye, but in addition the possession of photophores and the ability to produce light is a most important factor in this adaptation.

SUMMARY

1. Fourteen species of *Sergestes* have been taken in closing nets from the western part of the North Atlantic and the size and structure of their eyes have been related to the depth at which certain species occur and to the presence or absence of photophores.

2. *Sergestes mollis* is ordinarily taken below the photic zone and this species lacks photophores. The corneal portion of the eye of this form is smaller in relation to body size than is that of any other species studied. The eyes, however, are not so degenerate structurally as those of *Hymenodora glacialis,* an acanthephyrid having a similar vertical distribution.

3. *Sergestes tenuiremis, S. grandis, S. crassus and S. robustus* have been shown to possess organs which are probably photophores and these four species have the largest eyes in respect to body size of all which have been studied. Hence it may be concluded that the production of light by such an organism influences in some way the development of the eye. This agrees with the findings on the acanthephyrids.

4. The remainder of the sergestids studied which live within the photic zone have eyes smaller than those which possess photophores and in certain cases (*S. arcticus* and *S. armatus*) the pigmentation of the eye is quite unusual.

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THE QUANTITATIVE VERTICAL DISTRIBUTION OF MACROZOÖPLANKTON IN THE ATLANTIC OCEAN BASIN[1]

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During the summer of 1933 investigations were begun on the quantitative distribution of plankton with depth in deep water in the offing of Woods Hole, based on the volumes caught at different depths at levels two hundred meters apart (Leavitt, 1935). The present report is a continuation of this work. It also includes a partial analysis of the fauna which is responsible for variations in quantity at different depths.

Localities of Collection

The collections were made from the research vessel "Atlantis," at 9 stations, 100 to 300 miles south and east of Woods Hole (Fig. 1); precise localities and dates are given in Table I (p. 378).

Three of the ten stations (1733, 2263, 2462) were located in Sargasso Sea water, offshore from the Gulf Stream, one (1735) in the axis of the latter,[2] and six (1737, 1739, 2216, 2260, 2463, 2475) in the slope water inshore from the latter. These locations were chosen as representing oceanic conditions, all being well outside the continental edge, in deep water. One hundred and seventeen deep hauls were made, which may be dealt with from a quantitative point of view.

Methods

The material was collected in closing-nets, either 1 meter or 2 meters in diameter, operated as earlier described (Leavitt, 1935). The releasing device (Fig. 2) has subsequently been improved to overcome the difficulties which arose with earlier models. It is made of phosphor-bronze and is hinged so that it may be opened to place it (on the cable) around a clamp which is bolted to the cable to prevent the releasing device from sliding up and down. The toggle bars to which the bridles are attached are so shaped as to obviate any danger of their failing to swing out and drop the bridles, when released. The messengers, also, are hinged to facilitate placing them on the cable, and locked in place by a pin. The proper functioning of the net depends on the release of the toggle bars in the correct sequence by the messengers (Fig. 2). This depends not only on the second trigger being offset far enough to obviate any danger of its being struck by the first messenger, but also on the second messenger (cupping the first) being large enough in diameter and long enough to receive proper guidance by the towing cable. Experience has also shown that unless the messengers are bored somewhat oversize the vibration of the cable may impede their progress downward through the water.

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[1] Contribution No. 174 of the Woods Hole Oceanographic Institution.

[2] Following Iselin (1933) the term "Gulf Stream" is used to include not only the band of warmest surface water or that wherein the northeastward current is most pronounced, but the whole body of water, extending down to 2,000 meters, across which the isotherms and isohalines dip steeply in the inshore-offshore direction.

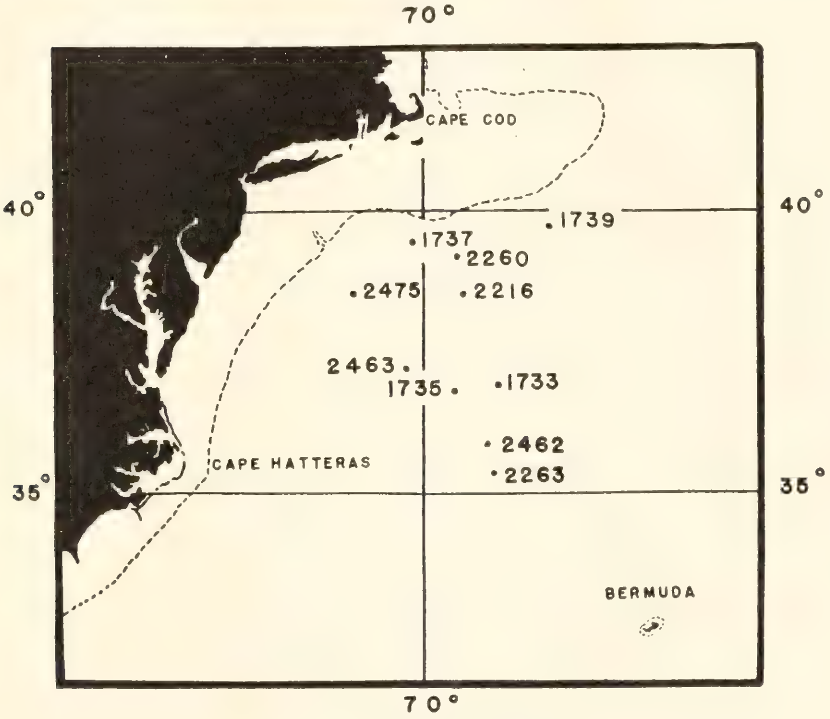

FIG. 1. Location of stations.

The releasing device and messengers are adapted for use in series and four nets were used, simultaneously, on the one cable.

Since there is no way of determining, from the condition of the nets themselves, on their arrival at the surface, whether they were open the full period of the tow, but only whether the proper bridles were released, we must turn to the consistency of the results for evidence in this respect. It will be seen in Table I that we usually found more than one rich level in the upper part of the water column, followed by two or more poor catches in mid-depths, with two or three somewhat more productive hauls still deeper. It is very unlikely that nets would consistently fish well at the shoaler levels, fail at the mid-levels, but work well still deeper down. Nor is it conceivable that hauls from the same depths would fail every time at the same depths at different stations in different years. Stations 2216, 2260, and 2462 are particularly significant in this respect, for they show a marked similarity in vertical distribution (Table I). These similarities may be considered strong evidence for the adequacy of our technique since the first two stations were made two months apart, and the third a year later, all in the same general water mass.

TABLE I *

Catches in cubic centimeters, calculated per 2 hours towing with a 2-meter net

Sta.	1735	1737	1739	2216	2260	2263	2462	2463	2475	Average
Date.	7/28/33	7/30/33	8/12/33	6/11/34	8/19/34	8/23/34	7/27/35	7/31/35	8/31/35	
Lat.	36°50′N	39°29′N	39°42′N	38°26′N	39°12′N	35°17′N	35°50′N	37°21′N	38°46′N	
Long.	69°16′W	70°14′W	67°04′W	69°05′W	69°13′W	67°28′W	68°39′W	70°26′W	71°38′W	
200M.	105	300	250	200	93	33	105	375	521	221
400.	50	280	255	18	105	84	33	200	122	105
600.	55	280	345	70	210	23	48	134	181	150
800.	50	225	270	500	510	78	64	57	115	207
1000.	33	125	150	45	10	75	42	54	148	77
1200.	16	20	23	20	5	75	18	51	140	41
1400.	37	57	27	55	90	35	4	35	8	39
1600.	60	93	30	300	25	25	35	24	29	69
1800.	80	125	45	70	3	8	10	9	0	31
2000.	100	160	60	0	40	15	20	11	0	45
2200.			105	10	60	38	17	5		
2400.			151	12		32	62	4		
2600.			185	4		6		7		
2800.			220	2		4		4		
3000.			256	13		0		2		
3200.			0			2				

* Positions and dates given are for the starting points of tows.

Another proof of the accuracy of the method is found in the fact that in a great majority of the cases where hauls were repeated at the same level at the same station, the variation in the amount of material caught was less than 30 per cent and often less than 10 per cent. In short, it seems that the methods employed were sufficiently reliable to yield consistent results.

The stated depths are as calculated from the length of wire outboard and its angle with no allowance for catenary. Failure to take account of the latter no doubt introduces an error, the magnitude of which is assumed to be not greater than ± 100 meters. In any case, it should be approximately the same for all hauls at a given level as care was taken to maintain a constant wire angle and control of the speed of the ship. The average speed of towing was close to two miles per hour in all cases.

The problem at hand being the difference in the productivity of the different levels of the sea, a volumetric system of analysis seems as significant as any other yet devised.

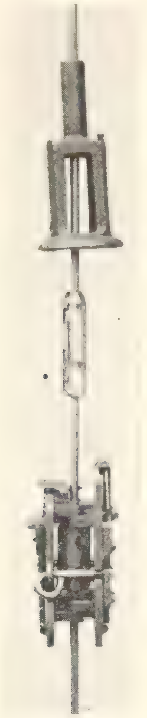

Fig. 2. Releasing device and messengers.

The total volumes of all the catches were first measured by the displacement method (Leavitt, 1935, p. 120), the volumes of the following groups then measured separately: fish, copepods, decapods, euphausiids, chætognaths, cœlenterates, salps, and "residue."

Since the duration of the tows at different stations varied between one and two hours and since a few of the early tows were made with nets of different sizes, all volumes have been reduced to the common standard of two hours towing with a 2-meter net.

In addition to the volumetric measurements, the euphausiids were all identified and counted. In some cases, when great numbers were caught, it was necessary to use a sampling method for counting closely allied species (Leavitt, 1935, p. 125). Some other groups[3] which were of particular interest in certain of the hauls have also been identified and counted, either in aliquot samples or in the entire catch, when the latter was small.

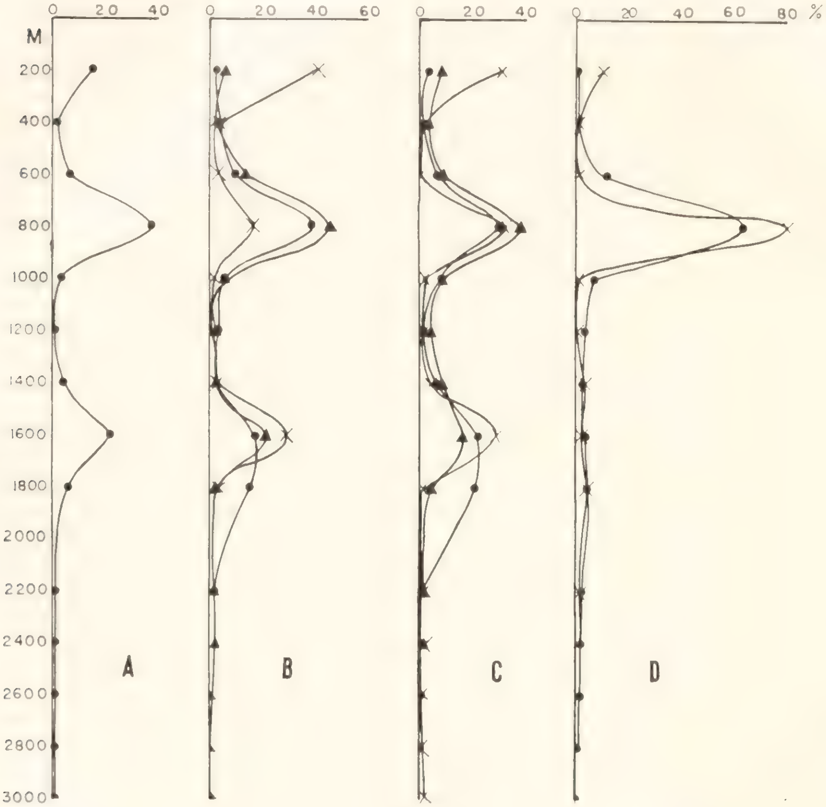

FIG. 3. Percentages of catch, at different levels at Station 2216. *A*. Percentage of total catch. *B*. Percentage of fish (●), copepods (▲), and euphausiids (×). *C*. Percentage of decapods (●), chætognaths (▲), and salps (×). *D*. Percentage of cœlenterates (●), and "residue" (×).

RESULTS

The most obvious feature of vertical distribution, illustrated by the foregoing tabulation, is that in spite of wide differences in detail from station to station, all stations agree in showing a peak of abundance at some level shoaler than 1,000 meters. And the validity of this generalization is corroborated by the fact that, on the whole, the percentual distribution of the catches of the different groups at a given station, parallels that for the total catch there, as illustrated by Fig. 3 (derived from Table I).

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[3] Drs. M. Sears, H. B. Bigelow, and F. Chace have assisted with the identification of copepods, cœlenterates, and decapods.

In some instances, it is true, the curves for single groups show seemingly large peaks at depths where none appear on the curves for the total volumes. This is accounted for by the fact that the percentage of a single group at one level may be great when the importance of that group in the total community is negligible. Such instances are, however, exceptional.

Table II

Station No.	Percentage taken above 700 meters per cent	Depth at deepest haul meters
1735	58	2,000
1737	93	2,000
	Percentage taken above 800 meters per cent	
1739 *	—	3,200
2216	60	3,000
2260	80	2,200
2263	40	3,200
2462	55	2,400
2463	68	3,000
2475	85	2,000

* At Station 1739, the only station at which there were a great many salpæ, over 94 per cent of the plankton caught was taken between 800 meters and the surface. If we subtract the volume of salpæ which constituted over 80 per cent of the total catch in four out of the thirteen hauls, approximately 74 per cent of the catch was taken between 800 meters and the surface, 26 per cent in depths greater than 800 meters.

The prevailing richness of the upper 800 meters, contrasted with the barrenness of the underlying stratum, is further emphasized by the following tabulation of the percentage of the total catch that was taken in the upper part of the water column (Table II).

It seems sufficiently established from Table II that from 40 per cent to over 90 per cent of the animals (by volume) were living in depths less than 800 meters, which is not surprising in view of the works of many early investigators. Within this upper zone the curves for individual stations differ widely, one from another, in the presence or absence of pronounced peaks (of abundance), and in the depths at which such peaks occur (Stations 2216, 2260, 2463, 2475, and to a lesser extent at 1739). Such peaks, in the upper levels, which include the photic zone, may reflect the recognized vertical migration of some surface animals, the gregarious habits of others, as well as the fact that the phytoplankton is confined to this layer, so that one would therefore expect a more erratic vertical distribution of the quantity of plankton there.

Strongly contrasting with the abundant peopling above, is the fact that at every station, the water contained much smaller volumes of plankton either at 1,200 meters, at 1,400 meters, or at 1,800 meters, than at any shoaler level, appearing on the individual curves as distinct minima. However, the comparatively barren zone still contained a small amount of plankton, composed largely of dead and decomposing corpses in many instances, but in several cases (particularly Stations 1735, 2216, 2260, and 2462), there were living copepods, chætognaths, decapods, and an occasional amphipod and cœlenterate present, though in very small numbers.

Perhaps the most significant results of our exploration is that in 8 out of 9 cases, at least one level still deeper down proved considerably richer again, in plankton, either as a distinct maximum (Station 2216), as two such maxima (Stations 2260, 2465), or as a progressive increase in abundance, extending downward through a stratum several hundred meters in thickness (Stations 1735, 1737, 1739). In the extreme case (Station 2462), the secondary maximum at 2,400 meters was in fact some sixteen times as rich as the overlying minumum; in another case (Station 2216), the tow was fifteen times as productive at 1,600 meters as at 1,200 meters.

Corresponding to this, the average catch, for all stations combined, was about 1.5 to 1.8 times as large at 1,600 meters as at 1,200–1,400 meters. And while averages for greater depths perhaps are not significant, because only four of the stations were worked deeper than 2,400 meters, calculation of what percentage of the catch for the entire column was made at different depths shows a similar relationship (Fig. 4), followed by decrease from 1,600 meters to 2,000. Deeper than this, the average percentages were consistently insignificant down to the greatest depths fished, except at one station (1739) where the stratum between 2,400 and 3,000 meters rivalled the upper 800 meters in richness. Even in that case, however, the deepest haul (3,200 meters) yielded nothing at all, although the net appeared to have fished properly. At two other stations (2263, 2463), also depths greater than 2,400 meters were practically barren, and conditions at Station

2475 show that, on occasion, this may apply at as small a depth as 1,800–2,000 meters (for details, see Table I, p. 378).

It is also significant—as bearing on its origin—that in every case the existence of a comparatively rich stratum at great depths, reflects, not only the relative volumetric abundance of the community as a whole, but also that of several individual groups of animals, as typified by the recorded volumes of fish, copepods, euphausiids, decapods, chætognaths, cœlenterates, salps, and "residue" at Station 2216 (Fig. 3). That these maxima represent widely diversified communities is further

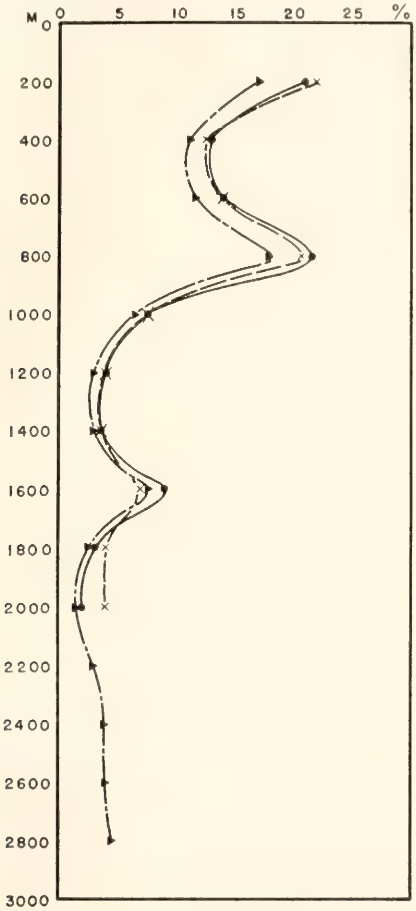

FIG. 4. Percentages of total catch to different levels based on data in Table I. ●, percentage at nine stations to 2,000 meters. ×, percentage at four stations to 2,000 meters. ▼, percentage at four stations to 2,800 meters.

proven by the large number of species of copepods and decapods represented in hauls at the depths in question, at representative stations, as follows:

Station 1735, 2,000 meters, copepods, immature *Metridia* sp., 75 per cent of sample, also *Metridia longa, Rhincalanus cornutus, Rhincalanus nasutus, Paraeuchæta norvegica, Paraeuchæta barbata.*

Station 1737, 2,000 meters, immature *Metridia* sp., 24 per cent of sample, also the species listed above, and *Lucicutia grandis,* and decapods, *Eucopia biunguiculata, Hymenodora* sp.

Station 1739, 2,800–3,000 meters, copepods, immature *Metridia* sp., 37 per cent of sample, also *Calanus* sp., *Candacia* sp., *Corycæus* sp., *Eucalanus* sp., *Euchæta* sp., *Euchirella* sp., *Gætanus* sp., *Lucicutia* sp., *Oithona* sp., *Oncæa* sp., *Phyllopus* sp., *Metridia lucens, Metridia princeps, Pleuromamma abdominalis, Pleuromamma gracilis, Pleuromamma robusta, Pleuromamma xiphias, Pseudochirella obtusa, Rhincalanus cornutus, Rhincalanus nasutus, Scaphocalanus* sp., *Scolecithrix danæ, Scottocalanus securifrons, Temora stylifera, Valdiviella insignis.*

Station 2216, 1,600 meters, copepods, immature *Paraeuchæta* sp., 51 per cent of sample, also, *Euchirella intermedia, Euchirella messinensis, Euchirella rostrata, Heterostylites longicornis, Metridia princeps, Paraeuchæta barbata, Paraeuchæta norvegica, Pleuromamma robusta, Pleuromamma xiphias, Pseudochirella notocantha, Rhincalanus cornutus, Rhincalanus nasutus;* decapods, *Acanthephyra purpurea, Ephyrina benedictæ, Eucopia* sp., *Gennadus* sp., *Hymenodora glacialis, Sergestes* sp., and mysids.

Station 2462, 2,400 meters, copepods, *Euchæta* sp., 20 per cent of sample, *Lucicutia grandis,* 11 per cent, *Paraeuchæta barbata,* 14 per cent, *Rhincalanus nasutus,* 17 per cent, also, *Euchæta marina, Gaidius* sp., *Megacalanus* sp., *Metridia princeps, Paraeuchæta norvegica, Pseudochirella paliata, Pseudochirella pustilifera, Rhincalanus cornutus;* decapods, 1 *Gnathophausia* sp., 1 *Parapasiphæ sulcatifrons;* cœlenterates, 1 *Halicreas papillosum,* 1 *Homœonema* sp., 6 *Periphylla hyacinthina.*

Station 2475, 1,600 meters, copepods, *Rhincalanus nasutus,* 30 per cent of total copepods (310), *Calanus* sp. (immature), and *Paraeuchæta norvegica,* together, 40 per cent; the remainder represented 15 other species, all of which have previously been found at similar depths.

Other hauls at the levels of the secondary maxima (Station 2260, 2,200 meters, Station 2263, 2,200 meters, Station 2462, 1,600 meters, Station 2463, 2,000 meters) similarly yielded from one to seven specimens each among the following cœlenterates and ctenophores, *Æginura grimaldii, Amphicaryon acaule, Atolla bairdii, Beroe* sp., *Chuniphyes multidentata, Halicreas minimum, Hippopodius hippopus, Homœonema* sp., *Pantachogon rubrum, Periphylla hyacinthina,* and *Rhopalonema funerarium.*

VERTICAL DISTRIBUTION OF EUPHAUSIÏDS

The euphausiïds have been chosen as a representative group of animals for numerical analysis for the reason that they are holoplanktonic and are worldwide in their distribution as a group, that they extend their vertical distribution to all depths of the ocean, that the species are not so numerous but what one may readily become familiar with them, and that little is known of their quantitative vertical distribution outside the continental shelves.

Evidence that our hauls contained a representation of species sufficiently broad to be accepted as typical of mid-latitudes, on the high seas, is that they yielded all but one of the 27 species recorded by Tattersall (1926) from the collections made in 1914 by the U. S. C. G. Steamer "Bache" in waters adjacent to and south of the positions where the present collections were made. And this one (*Thysanoessa microphthalma*) appears to be confined to lower latitudes of the Atlantic and Pacific, north and south (Hansen, 1915). On the other hand, our collection contains eleven which the "Bache" failed to find, namely, *Thysanopoda orientalis, T. obtusifrons, T. acutifrons, Euphausia pseudogibba, Thysanoessa parva, T. inermis, T. longicaudata, Nematoscelis atlantica, Stylocheiron affine, S. insulare.*[4]

Similarly, the present collections contain all the species reported by Hansen (1912) from the same general region, besides twelve others which he does not record there, although several of these are known to be widely distributed in the western Atlantic, and have been taken not far away; namely, *Thysanopoda tricuspidata, T. pectinata, T. obtusifrons, Euphausia pseudogibba, Nematobrachion flexipes, N. boöpis, Stylocheiron longicorne, S. abbreviatum, S. affine, S. maximum, S. suhmii,* and *S. insulare.* Seven of these are recorded from farther south by Tattersall (1926). Again, we find, in the "Atlantis" collection, all but two of the species reported by Ruud (1936) from southwest of Ireland, from Cadiz Bay, and from the Mediterranean. And one of these (*T. microphthalma*) had already been reported from the western North Atlantic by Tattersall (1926), while the other (*Nyctiphanes couchii*) is a coastal species, so far known only in the Mediterranean and the eastern Atlantic.

We may conclude from the foregoing comparisons that we are dealing with a good representation of the total euphausiïd fauna of the

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[4] *Meganyctiphanes norvegica* was also taken in several tows at stations 1739 from unkown depths.

TABLE III

Total numbers of specimens of euphausiids caught at different depths, at Stations 2216, 2260, 2263, 2462, 2463, and 2475 combined.

Depth	200	400	600	800	1,000	1,200	1,400	1,600	1,800	2,000	2,200	2,400	2,600	2,800	3,000
Number of hauls	7	8	8	7	6	6	6	7	5	6	6	4	3	3	2
Meganyctiphanes norvegica	37	258	3												
Thysanopoda orientalis			8	14	1	1			1			1			
Thysanopoda tricuspidata															
Thysanopoda pectinata		3	1	3											
Thysanopoda acutifrons				1											
Thysanopoda æqualis	8		1	61				1							
Thysanopoda monacantha		14	2	3											
Thysanopoda cornuta			1												
Thysanopoda obtusifrons											3				
Euphausia tenera	1	92	110	97				11			16				
Euphausia americana	2	10		61											
Euphausia krohnii	3,068	49	4	4	3	3					1				
Euphausia hemigibba	4	753	243	24	12			4			10				
Euphausia pseudogibba		17		69				12			14				
Euphausia gibboides	21														
Euphausia mutica			52												
Euphausia brevis	48			24				4				5			4
Thysanoessa gregaria	2,439	77	230		1	16									
Thysanoessa parva		2	262	1,941	466	699	926	2,804	91	88	1,540	480	1	1	4
Thysanoessa inermis		2													
Thysanoessa longicaudata	3	1,504	222	201	328		94								
Nematoscelis megalops	11,670	29,694	2,043	7		1		466			8				
Nematoscelis microps	82	231	92	62	24	307		23		2	7	2			

Table III (*Continued*)

Depth	200	400	600	800	1,000	1,200	1,400	1,600	1,800	2,000	2,200	2,400	2,600	2,800	3,000
Number of hauls	7	8	8	7	6	6	6	7	5	6	6	4	3	3	2
Nematoscelis atlantica			98	105											
Nematoscelis tenella	8	6	14	12											
Nematobrachion flexipes	9	12	18												
Nematobrachion sexspinosus		1	1												
Nematobrachion boöpis		3	3			6									
Stylocheiron carinatum	8	28													
Stylocheiron longicorne	555	347	14	5	2	1			1		8				
Stylocheiron elongatum															
Stylocheiron abbreviatum															
Stylocheiron affine	4							1							
Stylocheiron maximum		2	5	8											
Stylocheiron suhmii															
Stylocheiron insulare	19							2							
Bentheuphausia ambylops		1		4	5	7	3	3		2	2				
Thysanoessa sp.	2	1							3						
Thysanoessa sp. young								3							
Euphausia sp. young	4							3							
Nematoscelis sp. young	14														
Stylocheiron sp.	5	1													
Euphausia sp.		36		1		1			2	4					
Thysanopoda young				4											
Young Euphausids	89	4		2											
Nematobrachion sp.				1							6				
Nematoscelis sp.					1										

western Atlantic basin, especially in the fact that the list of species is a combination of northern with southern forms, to be explained by the latitudes at which the collections were made.

Earlier data (Leavitt, 1935, Tables II–V), and Table III of this paper show that the numbers of euphausiids taken was much smaller at great depths than at the shallower levels, for 86 per cent of the 76,790 specimens identified and counted were from tows between 800 meters and the surface, whereas only 14 per cent were from depths greater than 800 meters. These percentages are similar to those of the total volumes (p. 381).

Many of the euphausiids are notably gregarious animals and this fact coupled with vertical diurnal migrations may account for the unevenness of their occurrence in the upper layers.

In order to determine the relative importance of euphausiids as a whole in the community, the numbers of euphausiids per 50 cc. of plankton caught at each depth was estimated at four stations (Leavitt, 1935). While the numbers of euphausiids, like the quantities of plankton, increased on the average below 1,200 meters, the importance of this group in the total animal community decreased down to the deepest layer sampled, the number of euphausiids per 50 cc. of plankton being only about one-fourth as great at depths greater than 800 meters than in the upper layers.

There are few reliable data on the depth at which different species of euphausiids are to be found and Ruud (1936) regards temperature as one of the most important limiting factors of the distribution of the different species of this group. Since all but nine of the species recorded by him belong to groups most abundant between 200 meters and the surface, and since our shallowest hauls were at 200 meters, the data can hardly be compared with his. But among the nine species that he regarded as deep-sea forms, it is interesting to note that *T. gregaria* (regarded by Zimmer (1914) as bipolar) occurred abundantly at a depth of 2,000 meters at Station 1735, and *T. parva*, which did not occur in Ruud's material, may be regarded as even more persistently bathypelagic, on the basis of the present catches.

The decrease in numbers of euphausiids with increasing depth is accompanied by a corresponding decrease in the specific diversity of the group, for Table III shows only 18 species out of the total of 37, as occurring at all, below 800 meters and most of them in such small numbers (sometimes a single specimen) that only *Bentheuphausia amblyops* and *Thysanoessa parva* can be regarded as truly bathypelagic in these waters.

Thus, it seems sufficiently established, at least for this part of the ocean, and time of year, that euphausiids as a group reach their highest development in the upper 800 meters.

In the main, however, it is obvious, in common with Tattersall's (1926) experience, that the species in question show a deeper vertical range in the area investigated than in the eastern Atlantic and Mediterranean areas surveyed by the "Thor" (Ruud, 1936).

Discussion

Vertical Distribution

The precipitous variation in the abundance of a species in a given area has often been remarked. Michael's (1911, 1913) and Hardy's (1936) statistics as to the variation in numbers of chætognaths and other animals caught in a single place with successive hauls, and in closely proximate places, emphasize the fact that generalizations concerning distribution are extremely hazardous. Hence, we must realize the dynamic character of the plankton, and that attempts at a hard and fast terminology of the regions of vertical distribution are futile. It is only by continued observations over a period of time with a great many hauls that general ideas of distribution can be arrived at. Single hauls at separate localities can only be regarded as proof that what was caught was present at that particular time and place where the tow was made.

Nevertheless, it seems established by our investigations that in the region examined the abundant surface fauna decreases in quantity to a comparatively barren zone at a depth somewhere between 1,200 and 1,800 meters and that—in this particular region, at least—this minimum is followed by a subsequent increase in volume, at some greater depth.

Broadly considered, this is in line with previous knowledge, based largely on the work of Chun (1888, 1895), Alexander Agassiz (1888, 1892, 1902), Haeckel (1890), and Fowler (1898, 1904) that while pelagic animals live at all depths in the sea, with wide variety of species even at great depths, a much larger quantity occurs in the upper 800 meters or so than in deeper water.

Fowler's (1904) data on plankton from the Bay of Biscay is of particular interest in the present connection as the first detailed study of vertical distribution down to great depths in a restricted area, based on closing net hauls.

Subsequent investigators, including Esterly (1912), Murray and Hjort (1912), Michael (1913), Jespersen (1915, 1923, 1935), Hardy and Gunther (1936), Leloup and Hentschel (1935), Thiel (1935), Ramner (1935), and Bigelow and Sears (1937), have also recorded vertical variations in quantity for siphonophores, medusæ, copepods, fish, chætognaths, corycæens, and plankton as a whole. In none of these cases are the data of a sort to show whether or not there was any consistent increase in the volumes of plankton with increasing depth below the 1,200–1,800-meter level.

The plankton-poor layer encountered by "Atlantis" somewhere between 1,200 and 1,800 meters is presaged by A. Agassiz (1888) and others. The rich streak at greater depths, however, was unexpected, and the animals found at these greater depths are holoplanktonic, not benthonic, as illustrated by the assemblage collected at Station 1739 from a depth of 3,200 meters, where the total depth was 3,674 meters. In fact, there was no indication of the presence of benthonic animals, in any of our hauls, nor, in fact, would such have been expected for even the deepest tows were still far above the bottom.

In view of the changes in the physical and chemical characteristics of water with increasing depths, it is interesting, in the first instance, to correlate the volumetric distribution, vertically, of the plankton of the open sea with the density, salinity, and temperature at different levels in the slope water and in the Gulf Stream beyond.

Typical graphs (Fig. 5) for temperature, salinity, and density for the waters in which these investigations were carried on show no correlation between any of these environmental factors, and the depth distribution of the animals. Thus, the temperatures at all depths are well within the range in which we find plankton thriving at other times and places. The salinity variations are too minute for us to suppose that they have any appreciable effect on the plankton in these waters. Again, we find the same animals living throughout wider ranges of salinity than are presented here. The density does not show any indication of layering such as might account for an accumulation of material at any particular level between the surface and the bottom. In the present case, there is no apparent correlation between quantities of plankton, and either oxygen or light. Neither do any of these characters of the water show fluctuations below a depth of 800 meters corresponding to the existence of the plankton minimum at 1,200–1,800 meters, or to the subsequent increases in the volume of plankton at some greater depth.

The minimum oxygen in cubic centimeters per liter does not fall below 60 per cent saturation or about 3.5 cc. per liter in any of the water which was fished (Seiwell, 1934). And comparison between data for oxygen and numbers of animals caught at many of the "Meteor" stations as reported by Wattenberg (1935), Spiess (1932), and Hentschel (1932) shows that an abundance was caught in water where the oxygen content was much lower than at any level in the waters with which the present investigations are concerned. We must, therefore, conclude that there is ample oxygen to support planktonic communities at all depths there.

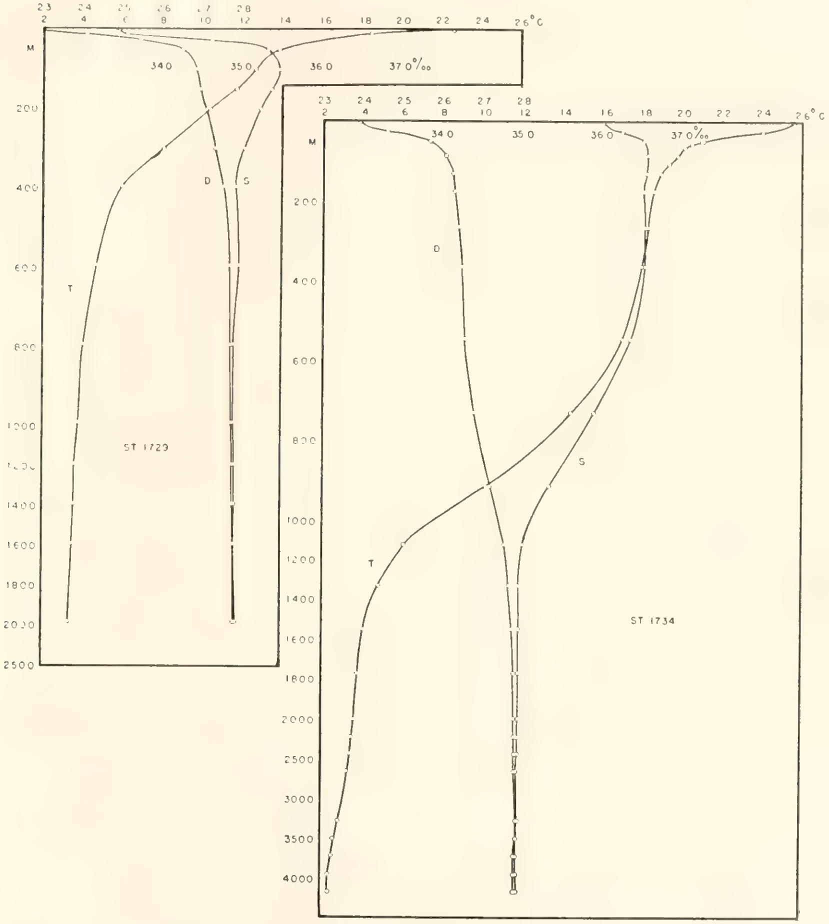

FIG. 5. Temperature, salinity, and density at "Atlantis" Station 1729, typical of slope water between the Continental Shelf and Gulf Stream and at Station 1734, typical of Sargasso Sea water east of the Gulf Stream, in the area of these investigations. *T*, temperature in °C. *S*, salinity in parts per mille. *D*, density (specific gravity minus one, times one thousand).

Among other possible causes for such vertical variations in abundance of plankton as do occur in deep water, the following possibilities come to mind:

(*a*) Dead and decomposing animals sifting down from the surface are sometimes caught in deep hauls. This happens oftenest with *Iasis zonaria,* the firmest bodied salp, pieces of which are found among the other dead and decomposing animal matter in deep hauls. Other salpidæ, also, contain cellulose which might be expected to disintegrate slowly.

(*b*) An occasional large medusa, *Periphylla hyacinthina* or *Atolla bairdii,* or some large deep-sea decapod, may rarely be responsible for a relatively voluminous catch at a great depth.

(*c*) A well developed bathypelagic fauna may—for some undetermined reason—find some particular deep levels more suitable as an abode than the levels above or below. The general identity of the animals taken in our tows shows this not to be the case.

(*d*) Sinking water masses from more moderate depths, in somewhat higher latitudes may bring their planktonic communities with them and so be responsible for richer strata below poorer, farther south. This last seems the most probable explanation for such a distribution in the present case, because species of decapods, copepods, cœlenterates, and euphausiïds occurring at moderate depths somewhat farther north have been prominent in the catches where total volumes have revealed the existence of secondary maxima, deep down. This, for example, applies to *Calanus finmarchicus, Metridia longa, Paraeuchæta norvegica, Rhincalanus nasutus, Rhincalanus cornutus, Pleuromamma abdominalis, Pleuromamma gracilis, Pleuromamma robusta, Pleuromamma xiphias,* among the copepods, several species of Gennadus, among the decapods, and *Thysanoessa gregaria,* among the euphausiïds.

It is likely that the animals belonging in this category are able to maintain themselves even in the deepest water layers for a considerable length of time, and that great depths may constitute the outskirts of the areas which they regularly populate. Lack of light may not harm them. Dr. C. B. Wilson has informed me that his examination of copepod material brought back from the Byrd Antarctic Expedition showed that many (including larval forms) were taken from under the ice, where it was totally dark, and far from open water. The " Terra Nova " Expedition also obtained many thousands of specimens under the ice (Farran, 1929). In short, communities may maintain themselves for a long time after sinking. We may have a biological loss, akin to that of the animals and plants that drift north and to their deaths in the surface waters of the Gulf Stream every summer.

While examination of vertical distribution was our primary objective, one phase of the horizontal picture deserves at least passing mention, namely the poverty of the catches in the Sargasso Sea water and in the

Gulf Stream, as defined on p. 376, contrasted with the slope water, the average catch (to 2,000 meters) being only 415 cc. in the first of these regions, 586 cc. in the second, contrasted with 1,283 cc. in the third. This regional gradient is of the same order as reported by Jespersen (1923, p. 8, Fig. 23) for average volumes for the upper 600 meters farther to the south, and is also in line with the prevailing poverty of the Sargasso Sea region, subsequently demonstrated by him (Jespersen, 1935, Figs. 27, 28).

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A RECONSIDERATION OF THE EVIDENCE CONCERNING A DORSO–VENTRAL PRE–ORGANIZATION OF THE EGG OF CHÆTOPTERUS

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In a former paper (1937) the question of the presence of a dorso-ventral axis in the unfertilized egg of *Chætopterus* was discussed. The argument rested in part on the assumption that the large germinal vesicle is excentric as in many other eggs, but here both laterally as well as axially excentric. Since, in eggs that have come to rest in a single layer on the bottom of a dish of sea water, the pole lies, as a rule, not at the top of the egg but more or less at the side, and since the germinal vesicle appeared, then, to lie symmetrically with respect to the circumference of the egg as seen from above (as in Fig. *A*), and since the first cleavage passes through the pole and in most cases through the uppermost side of the egg, it seemed to follow that there is a dorso-ventral predetermined orientation in the unfertilized egg.

Later, when I came to examine sections of the egg, it could not be visibly demonstrated that the germinal vesicle lies nearer the pole, i.e., in an excentric position with respect to the polar axis. It became evident then that the problem of the location of the dorso-ventral axis called for a closer examination. This examination I have made during last summer. The new evidence shows that the former argument is insufficient to establish the conclusions there presented.

If, then, the germinal vesicle does not lie excentric in the polar axis or excentric to that axis (as in Fig. *A*), it remains to consider other possible relations.

If, for example, the nucleus lies in the center of the egg (as in Fig. *B*) then when the pole lies above (or below) the equator of the egg the nucleus will again appear as seen from above to be in the center of the egg, and it is not possible to distinguish on this evidence alone, between *A* and *B*. There is a third possibility, viz., that the germinal vesicle lies excentrically in the polar axis (Fig. *C*) but not excentric to that axis. If this were true, then the egg, seen from above, would, when it lies with its polar axis oblique to the vertical, show the germinal vesicle slightly excentric to the circumference of the egg, but this does not fit in with the new or the old observations.

Should the pole of the egg come to lie near the uppermost part of the egg, as the egg settles down on the bottom of the dish, as indicated by any one of the ring of dots in Fig. *D*, then, the first cleavage plane, that always passes through the pole and the center of the egg, will be an approximately vertical plane. If the pole lies somewhere between the top of the egg and the equator, as in Fig. *E*, the first cleavage plane may be more or less oblique to the vertical, but will appear to cut through the upper surface of the egg in the great majority of cases. But if the pole of the egg lies at or near the equator, i.e., if the polar axis lies nearly in the horizontal plane, as in Fig. *F*, then the first cleavage plane should appear horizontally as well as vertically and also in all intermediate positions. On the basis of chance alone there should be as many vertical as horizontal first cleavages when the polar axis lies horizontally. Such

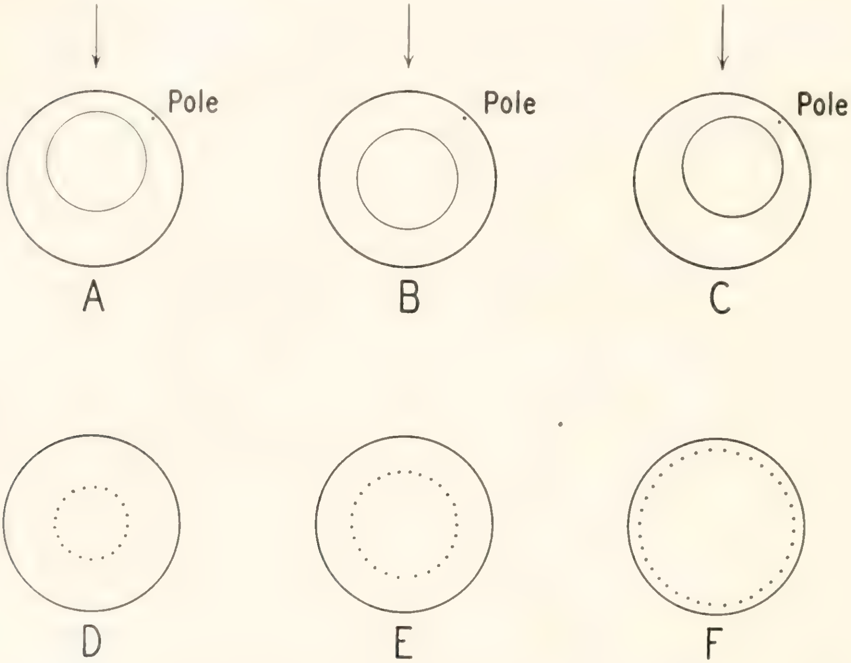

FIG. 1. *A*, diagram of egg showing nucleus excentric on primary axis and excentric to that axis. The arrow indicates the expected view as seen from above. *B*, diagram of egg with nucleus in center. *C*, diagram of egg with nucleus excentric on primary axis, but not excentric to it. *D*, egg with pole uppermost, as indicated by one circle of dots. *E*, same with pole sideways between top of egg and horizon. *F*, same with pole at or near horizon.

eggs should furnish critical evidence concerning the point at issue, viz., whether, as I assumed, the eggs come to rest with the dorso-ventral axis vertical.

As stated above, sections of eggs, killed at once after removal to sea water, showed a nucleus irregular in outline lying at or near the center of the egg. If it is excentric, the excentricity is so slight as to be negligible, and certainly would not be observable in the living egg. Since these eggs had been taken out in sea water and killed " at once," it was possible that the irregular outline of the nucleus was due to the action of the sea water which might make its excentricity difficult to detect. In fact the nucleus breaks down to form the polar spindle very soon after removal of the egg to sea water.

A new set of eggs was then preserved in the summer of 1937, without coming in contact with sea water, in four different killing fluids (Flemming, Bouin, picro sulphuric, boiling water) and sectioned and stained. In some cases the eggs became free in the preservative; in other cases they remained in the tied-off parapodium. The preparations showed that the parapodial eggs lie several layers deep on the string or band from which they arise, and, owing to crowding, have many different shapes; but even those in the outermost layers are very seldom perfectly spherical. In none of them is the nucleus obviously excentric. The nuclear wall conforms in shape somewhat to the shape of the egg, and is not often perfectly spherical. After different preserving fluids (and subsequent reagents prior to imbedding), the shape of the nucleus is to some extent affected, but is seldom perfectly spherical. It follows then that when removed to sea water the irregular outline of the nucleus is not due to the absorption of water by the egg (as the egg assumes a spherical shape), but traces back to the parapodial eggs.

An examination of the living eggs as seen from the side was also made. The eggs were placed in a drop of sea water on a slide (ringed with vaseline) across which a thread had been stretched. The preparation was held in a vertical position and examined under the microscope. If the nucleus lies in an excentric position on the polar axis this should become visible in some, at least, of the eggs, but no excentricity was observed. It is true that the outline of the nucleus of the living egg is not sharply defined, but nevertheless were the nucleus excentric this should be apparent, which is not the case. Of course, in a confined space (but not compressed) the eggs might not have time to orient as they fall onto the thread. Nevertheless, some of the eggs should show the excentricity of the nucleus if it were present.

A more critical examination of the position of the first cleavage was then made. It was found, as expected, that in most of the eggs the first cleavage was approximately vertical. These eggs had settled to the bottom of the dish containing sea water and were not covered. In an unexpected number of eggs the lobe appeared later on the upper surface, proving that in some eggs the polar hemisphere was down. Special attention was paid to those eggs in which the clear area (containing the pole) lay near the horizon, so that more eggs of this kind were selected. Since the polar area is difficult to see if it lies exactly above or below but easily seen if it lies nearer the equator, a larger number of eggs with an oblique polar axis (nearly horizontal) would be those selected for examination. In a total of 111 eggs recorded there were 58 in which the first cleavage was vertical, i.e., through the top of the egg; there were 13 in which it was oblique; and 40 in which it was horizontal or approximately so. In about half of the latter the smaller blastomere was above (24 cases), and in half the larger one was above. Adding together the oblique (13) and the horizontal cleavages (40) gives a total of 53 eggs. In other words, in this selected group of eggs about half of the cleavage planes were not vertical but oblique or near the horizontal plane.

For comparison with these figures another count of *unselected* eggs in the first cleavage stage was made, recording those in which the first cleavage was vertical and those in which it was horizontal. In 120 eggs all but six showed the plane vertical or nearly so; three of the six had the large cell on top and three the smaller cell.

Since, as explained above, vertical cleavages should predominate on chance alone, as they do, and since, as anticipated, some of the eggs in which the pole lies at the side should show oblique or horizontal cleavage, it follows that the original assumption that a definite side of the egg lies above is not established, but is the expected outcome of the relation of the cleavage plane to the pole.

A check on the number of eggs in which the pole lies below the horizon is obtainable in two ways in addition to observation of the polar bodies, which is often difficult. After the second cleavage the configuration of the four blastomeres is such, owing to the broad cross-furrow at the antipolar hemisphere, that the two poles can be distinguished from each other. The other method of locating the pole and the antipole is from the position of the yolk lobe at the time of the first cleavage, when it is either up or down. When it lies under the egg it is not often seen. In the records there are cases where the lobe was above, hence the pole was down, showing that it may sometimes lie below the horizon, i.e., such eggs have not oriented to gravity, but the observations were not systematically carried out, hence the ratios of cases where the pole is up or down cannot be definitely stated.

The evidence discussed above does not, of course, disprove the view that before fertilization there is a dorso-ventral organization of the egg. Whether there is such an arrangement still remains an open question to be examined by other methods, one of which should be a study of the relation of the first cleavage plane to the point of entrance of the spermatozoön. In addition to an earlier examination of this relation by Morgan and Tyler (1930) fifty-three new cases have been studied and will be described in another paper.

It remains to be discussed whether the polar hemisphere is lighter than the antipolar, which seemed to be demonstrated by the centrifuging experiments described in my former paper. The argument for this assumption was based both on the position of the pole when the eggs are centrifuged before the polar bodies are formed, and also on the orientation of the egg in the machine after the first or second polar body had been extruded. The evidence showed that while most eggs orient with the polar region toward the center of rotation, the orientation is by no means exact. The higher degree of orientation on the centrifuge than of orientation to gravity in a dish of shallow water is not surprising since the former force is greater than the latter, and also since the eggs, falling through a longer space, have more time to orient before reaching the bottom of the centrifuge tube than have the eggs in a dish of water. However, unless the eggs are centrifuged over a gum-solution on which they lie in a single layer, this inference does not hold entirely, since if many eggs are centrifuged in sea water, and come to be packed at the bottom of the tube, a considerable amount of shifting of the eggs may take place as they become crowded together.

If, then, it be admitted that the majority of eggs turn on the centrifuge with the polar (or animal) hemisphere toward the center of rotation, still this will not explain why the pole of the egg is as a rule excentric to the stratification. One possible explanation suggests itself, namely, that there is a ring of material around the pole that is lighter than the pole itself. This may seem improbable and is not demonstrated by the evidence at hand. The following hypothesis may seem more plausible. When centrifuging begins, the eggs lie at random with respect to the axis of rotation. Some of them will have their polar hemisphere more or less in this axis, others will have it at the side, and others will have the polar hemisphere away from the center of rotation. Under the influence of the centrifugal force all of the eggs will begin to turn with the polar hemisphere toward the axis of rotation. Now if the redistribution of the lighter and heavier material within the egg begins while the egg as a whole is rotating, its rotation will cease as soon as the redistribution of the materials brings a region lighter than the pole toward the center of rotation. Hence many of the eggs may have the oil cap to one side of the true pole, viz., those that were not centered when the movement of the material within the eggs began to take place. This suggestion could possibly be tested by allowing the eggs to orient to gravity before centrifuging. More of them would then be expected to show the pole in the center of the oil field.

In connection with the problems discussed above the question remains as to whether or not the egg shifts its position after settling on the bottom of the dish. The fact that the egg undergoes a definite series of changes in shape might seem to make it possible that a partial rotation might take place. For example, when the first polar body is extruded the polar cap flattens and the egg becomes broader, i.e., biscuit-shaped. It then rounds up and remains spherical until about five minutes before the cleavage is due, when it becomes pear-shaped with the small end of the pear at the pole. Again it becomes spherical and flattens over the polar area as indications of the cleavage begin to appear. At this time it elongates laterally, i.e., it becomes biscuit-shaped as the lobe appears exactly opposite the polar bodies. At first it can not be foretold which side will become the smaller blastomere and which the larger, but very soon the difference becomes apparent in side view and this continues until a large and a small cell are formed. The egg is much extended laterally at this time. The two blastomeres next come together, but the egg does not become quite spherical. During the first of these changes in form the eggs do not turn over as shown by the constant position of the polar bodies, but as the two blastomeres come together, the first cleavage plane may appear to be more nearly vertical than during the division. Evidently the jelly around the egg adheres sufficiently to the dish to keep the egg in place. Moreover, the egg cannot rotate within the surrounding membrane. In fact, the latter adheres so closely to the egg that it indents with the cleavage furrow. It consists of a transparent inner layer—which may be a gelatinous layer between the egg and the outer jelly. When the polar bodies are extruded they push the inner membrane before them. Before and during this time the membrane is thrown into a series of corrugations over the polar region. Similar corrugations appear later over the region where the lobe is about to appear. The meaning of this folding of the membrane is not apparent, since it might have rather been supposed that the membrane would be stretching over these regions. Possibly surface movements of the protoplasm toward the region of activity may cause the wrinkling of the membrane.

THE RELATION BETWEEN ENTRANCE POINT OF THE SPERMATOZOÖN AND BILATERALITY OF THE EGG OF CHÆTOPTERUS

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In a preceding paper (*Cytologia,* 1937) we examined the relation of the entrance point of the spermatozoön to the cleavage planes of *Chætopterus, Cumingia,* and *Nereis.* In *Chætopterus* there was a coincidence of 41 per cent between the entrance point and the first plane of cleavage. In *Cumingia* the coincidence was 79 per cent. In *Nereis* it was 51 per cent. The failure to obtain 100 per cent coincidence may be due to errors of observation or to other factors being involved in determining the first cleavage plane.

We have made some additional observations during the past summer on *Chætopterus* since its eggs gave the lowest percentage of coincidence, and since a larger number of observations seemed desirable. Out of 53 new observations there were 24 cases of fairly close agreement; in 7 cases the divergence was less than 45 degrees, and in 22 cases it was more than 45 degrees. Approximate concidence was 45 per cent, as compared with 41 per cent of the old data. This absence of exact agreement led to an examination of other possible relations. When the entrance point is plotted on the egg in the two-cell stage it is found in the great majority of cases that it lies on the side of the egg where the smaller blastomere (AB) is found. In the new data there were 29 cases on the AB side, and only 9 cases on the CD side. In the older data there were 47 cases on the AB side, and 21 on the CD. But since the CD side has twice the surface area of the AB side the difference is more significant.

In these data cases of exact coincidence between the entrance point and the first cleavage plane are, of course, omitted. When the entrance point is plotted on the four-cell stage of these same eggs it is found that, in the majority of cases, it lies on the B cell, namely in 20 cases on the B; in 3 cases on the A; in 3 on the C; and in two on the D cell. In the new data it lies on the B cell in 15 cases, in 9 cases on the A, in 7 cases on the C, and 2 on the D cell. Cases of coincidence with the first cleavage plane give 11 with D opposite the sperm and 2 adjacent.

Taking these results as a whole, it is evident that the entrance point is on the AB side in by far the great majority of cases, and tends to be opposite the D cell. This may mean either that there is a preferred side for sperm entrance, or that the point of entrance determines as a rule the AB side.

In the previous paper referred to a similar result was observed in the eggs of *Cumingia*. The D cell consistently lies opposite the side on which the sperm enters. This occurs where the first cleavage plane coincides with the entrance point, and it follows from the relation between the type of second cleavage and the position of the AB blastomere with respect to the entrance point at the 2-cell stage. In *Nereis* the same result was obtained when the cases of coincidence of the first cleavage and the entrance point are considered. In 28 cases the D formed on the side opposite the entrance point and in five on the same side.

The method followed in these as well as in the former experiments is not well suited to determine whether or not there is in reality a preferred side of the egg at which a spermatozoön is more likely to enter. The eggs, to which a dilute sperm suspension is added, lie between the cover slip and the glass slide (without compression), and the spermatozoa, that come into contact with the egg almost immediately, are watched. It is by no means the case that the first spermatozoön, that makes contact with the egg, is the one that enters, but whether this is because the spermatozoön fails to make the proper head-on contact, or whether it has made contact with a side of the egg that is less easily entered cannot be stated. It is true that, with some experience, one can generally tell at once whether a given sperm is likely to enter or not. If it is about to enter its tail ceases to vibrate and stands out stiffly at right angles to the surface, but if it fails its tail continues to vibrate for some time. Only those spermatozoa that lie at the edge of the egg can be clearly observed. Those that enter above or below the rim of the egg cannot be seen. Hence, there is necessarily a certain amount of selection, but this in itself does not seriously affect the situation since the selection of the eggs observed is a random one. It might be possible, by pushing each egg halfway into a small glass tube closed at one end, that has the same diameter as the egg, to determine whether the spermatozoön enters only some of the eggs, namely, those with the preferred side exposed, or whether if they do enter all the eggs the entrance is delayed in approximately half of the cases. In this way it might be possible to find out if there is a preferred side, in other words, whether there is a predetermined dorso-ventral axis.

SOME EXPERIMENTS UPON TEMPERATURE ACCLIMATIZATION AND RESPIRATORY METABOLISM IN FISHES[1]

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Introductory, Procedure, etc.

In a preliminary communication from this laboratory (Sumner and Wells, 1935), experiments were described in which fishes of varying temperature history were subjected to a number of lethal agents and anesthetics. The most important conclusions drawn from these experiments were stated as follows:

" Fishes which had been kept at widely different temperatures, when tested at these temperatures, were found to differ markedly in the rate of their visible respiratory movements. . . . Other things equal, these are higher at higher temperatures than at lower ones.[2] . . . When fishes which had become acclimatized to high and low temperatures, respectively, were transferred to a common temperature (usually an intermediate one), those from the warmer water displayed a lower respiratory rate and a lower susceptibility to the anesthetics and lethal agents than those from the colder water. Thus the differences in physiological activity which had been originally induced by acclimatization to these differing temperatures were completely reversed when the fishes were brought to a common temperature. After this transfer, it was the former " warm " fishes which had the lower respiratory rate etc. and the former " cold " fishes which had the higher. Fishes kept at intermediate temperatures remained intermediate.

" Acclimatization to a high temperature appears to consist, in part at least, of a change whereby an initial great increase in metabolic rate is followed by a regulative process, with continued sojourn in warm water, and, conversely, acclimatization to a low temperature involves a similar process working in the opposite direction."

Somewhat earlier N. A. Wells (1935*a*), from actual determinations of oxygen consumption in *Fundulus parvipinnis,* had concluded that

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[1] Contributions from the Scripps Institution of Oceanography, New Series, No. 9.

[2] The further statement in the original text relative to higher susceptibility to urethane at higher temperatures has not been substantiated by our later work.

" the rate of metabolism of fishes at any given temperature is dependent upon the temperature of the water to which they have been [previously] acclimatized."

That the resistance of a living organism to cyanides and some other poisons varies inversely with the temperature was established by Child for planarians and other invertebrates (Child, 1913). Geppert (1889) is said to have proposed the view, now generally accepted, that the cyanides act by preventing the tissues from utilizing oxygen.

The occurrence of a certain amount of physiological adaptation in the effects of temperature upon metabolic rate was pointed out by Miss Behre (1918), working upon *Planaria dorotocephala*. In a discussion of experiments in which the periods of acclimatization ranged from three days to three months, she states: " Worms tested immediately after they have been put into a higher temperature than that at which they have been living for a shorter or longer time show greater susceptibility to cyanide than those which have been living indefinitely at a higher temperature; those tested immediately after they have been put into a lower temperature than that at which they have been living for a shorter or longer time show a lower susceptibility to cyanide than those which have been living indefinitely at the lower temperature."

Our discussion has thus far related to the effect of temperature upon metabolic rate, and the only result of acclimatization thus far considered has consisted in a fall in this rate, following an initial rise, or a rise following an initial fall. These changes in metabolic rate have been detected by observing the relative susceptibility to KCN or certain other poisons. Another type of acclimatization has to do with the effects of subjection to moderately high or low temperatures upon subsequent resistance to heat and cold, when these are used as lethal agents. That this latter class of effects is quite distinct from the former one will appear probable from facts to be reported below.

Among the earlier experiments in acclimatizing vertebrate animals to relatively high temperatures, those of Davenport and Castle (1895) are so familiar that a brief reference to them is sufficient. By subjecting toad tadpoles for four weeks to 24°–25°, instead of 15°, at which the controls were living, the temperature necessary to produce heat-rigor was raised by 3.2° C.

Loeb and Wasteneys (1912) conducted some highly interesting experiments upon *Fundulus* (presumably *F. heteroclitus*). These experiments were largely concerned with the effects of various salts upon heat resistance. But the authors also sought to determine the maximum temperature which the fishes could tolerate, if transferred suddenly from their natural medium (10° to 14° at the time); " how long it takes to immunize the fish against the harmful effects of a sudden transfer to a temperature of 35° C"; how long this immunity would last after return to cooler water, and some other matters. Certain of the results of Loeb and Wasteneys will be referred to later. For the most part, their paper does not report the numbers of fishes employed (where stated, they are very small), nor the variability of the individual records, so that the reader is left in doubt as to the statistical certainty of some of the results.

A rather extensive series of experiments on temperature acclimatization was conducted by Hathaway (1928) upon several species of fresh-water fishes as well as upon toad tadpoles. Hathaway's procedure consisted chiefly in keeping the animals for various periods at 10° and 30°, and then determining their mortality rate throughout various periods (1 minute to 24 hours) at temperatures ranging from 30° to 40°. "Continued exposure to high or low temperatures progressively raised or lowered the limit of tolerance of each species," a statement which is abundantly supported by his tables and histograms. No attempt was made, however, to relate the period of acclimatization in any quantitative way to the resulting degree of resistance.

The results herein reported are the outcome of experiments conducted by the authors throughout about a year past, in the course of which nearly three thousand fishes were employed. Except in one minor experiment, these fishes were gobies of the species *Gillichthys mirabilis* Cooper.

Our experimental material was kept in tanks of running sea-water at constant temperatures.[3] Tanks were maintained throughout at 10°, 20°, and 30° C., and for part of the time others were maintained at 15° and 25°. Prior to experimental treatment at high or low temperatures, all fishes were kept for some weeks or months at the intermediate temperature (20°). In some of our tables and charts, fishes from the 10°, 20°, and 30° tanks are designated as "C," "I," and "W," respectively.

The problems which have concerned us fall into two main groups as indicated in the preliminary discussion: (1) the effects of the subjection of fishes to higher or lower temperatures upon their respiratory metabolism; and (2) the effects of this sort of conditioning upon their subsequent resistance to heat or cold, when these were employed as lethal agents.

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[3] Except on infrequent occasions, the temperatures were held to within 0.5° of the figures stated, by thermostatic control or otherwise. Owing to failure in the control mechanisms, water in the 10° tank occasionally exceeded or fell below this figure by several degrees. The temperature of the 30° tank was less subject to such perturbations, though it fell some degrees below the required temperature on several occasions. It never rose above this, however, except very early in the course of these experiments.

(1) Some of the relations between this sort of temperature conditioning and respiratory metabolism were investigated by N. A. Wells (1935*a*, 1935*b*, 1935*c*) in this laboratory some years ago, through the direct measurement of oxygen consumption in *Fundulus parvipinnis.* In the present studies, no such direct measurements have been made. We have, however, tested some of these relations abundantly in other ways.

For this purpose, fishes of varying temperature history have been subjected to two principal lethal agents: KCN (0.001 M in sea water) and boiled sea water, containing about 5 per cent of the oxygen normally present. Since death from cyanide, as already stated, is due, in part at least, to asphyxiation, the rate of death from either of these lethal agents may be taken as a measure of respiratory metabolism.

In using each of these agents, our tests were of two sorts. The fishes were either (*a*) tested at the temperature, high or low, to which they had been acclimatized; or (*b*) they were tested after they had been returned for varying periods to the water of intermediate temperature (20°) from which they had been taken at the commencement of the experiment. In the latter case, the relations were also investigated between the duration of conditioning and the duration of the persistence of the induced effect.

In a limited number of experiments, the fishes of various temperature histories were subjected to the anesthetic urethane. The results from these experiments were less intelligible than those from other lethal agents, and the relation, earlier suggested (Sumner and Wells, 1935), between metabolic rate and susceptibility to this drug, now seems rather doubtful.

(2) Under the second head, we have chiefly sought to determine (*a*) the relation between the duration of previous conditioning and the extent of the subsequent resistance to heat (i.e. how high temperatures were lethal to the fishes), and (*b*) the relation between the duration of conditioning and the persistence of the effect, when the fishes were returned for varying periods to their original temperature.

In these experiments with lethal heat, our procedure has consisted in placing small lots of the fishes under comparison simultaneously in separate screen cages, immersed in a common tank. In any given set of experiments, the temperature at the outset was brought to the same level (most frequently 37.6°), before introduction of the fishes. It was thereafter raised at an average rate of about 0.1° in two minutes. Record was kept of the moment when each fish succumbed, and of the temperature of the water (to 0.1° or less) at that moment. Thus, it is evident that the temperatures recorded as lethal and the times recorded as the duration of resistance have no absolute significance. Their significance is entirely relative to the conditions of these experiments. In most of our experiments, there was little or no overlapping between lots of fishes having different temperature histories. This, although the mean lethal temperatures for such lots might differ by less than one degree.

Some tests were also made with a view to discovering the causes of certain unaccountable differences in our experimental results from lots of fishes which might have been expected to agree completely with one another. In general, none of the suspected causes were found to be responsible for these incongruities. The possible influence of differences in size, for example, was tested, despite our customary endeavor to select fishes of the same mean size for two lots under comparison. As will be pointed out below, size, within the range of differences which concern us here, could not have been a responsible factor in the matter, although size differences of sufficient magnitude were found to affect the respiratory rate and the rate of death in boiled water.

We likewise tested the possible effects of differences in the recency of feeding, and of minute differences in the concentration of the KCN solution. It was found that recency of feeding—even feeding within 24 hours—did not affect the resistance of the fishes to cyanide, while differences in the concentration of the solution as great as 2 per cent had no recognizable effect.

In all of our experiments, the value to be determined was the relative resistance of a given lot of fishes to the lethal agent or anesthetic which was employed. This resistance was measured by the time which elapsed before each individual "succumbed." It was consequently necessary to adopt a uniform criterion of "succumbing," in other words, a definite end-point which could be regarded as comparable throughout a given series.

The criterion adopted was necessarily different for the different types of experiment. In the case of lethal heat, the matter was simple. The end-point adopted was the time when the fish ceased to make any respiratory movements or other visible movements, spontaneous or induced. This time could ordinarily be recorded with an accuracy of less than half a minute. The figure recorded did not, however, necessarily represent the time of actual death, since many of these individuals recovered if transferred promptly to cooler water.

Where extreme cold was the "lethal" agent employed, a satisfactory end-point was more difficult to detect, since ready responsiveness to sharp mechanical shocks persisted long after all spontaneous movements ceased. Although our criterion was changed somewhat from one experiment to another, it was constant within any one experiment, so that some of the results are highly instructive.

In experiments involving asphyxiation (KCN and boiled water) the actual death of the fish was revealed by a more or less prolonged convulsive seizure, which was readily recognizable in most cases. More often, this commenced rather dramatically, after some minutes of complete quiescence, beginning with a vigorous flexure of the body, and passing into a characteristic series of quivering and twitching movements of the mandible, opercula and fins. The length and intensity of this convulsion were inversely proportional to the temperature, its duration in KCN solution averaging about 6 minutes at 10°, 3 minutes at 20°, and 1 minute at 30°. At the latter temperature indeed, it was frequently so brief or so inconspicuous that it could not be observed at all. The time of the commencement of these death throes has been adopted as the end-point in experiments with KCN and boiled water.

TABLE I

Mean times of survival in KCN at various temperatures, after three days accommodation to these, and relative rates of respiratory metabolism on which KCN curve in Fig. 1 is based. The last figures are the quotients obtained by dividing the reciprocals of the various times by the reciprocal of the time at 10°.

Number of Fishes	Temperature of Acclimatization (±)	Actual Temperature in Bowls during Experiment	Times of Survival		Relative Metabolism
			Mean	Range	
	°C.	°C.			
20	10	10.7	308.5	241–375	1.0
19	15	15.6	184.0	153–221	1.7
20	20	20.4	109.2	89–139	2.8
17	25	25.0	64.4	56–78	4.8
20	30	29.5	42.4	34–52	7.3

In experiments with urethane, choice of an end-point was a more difficult matter, especially at low temperatures, a fact which detracts from the value of many of our experiments with this drug.

In addition to records of their relative resistance to lethal agents and to urethane, we have records of the rate of the respiratory rhythm of many of our lots of fishes. The value of these last, though real, is limited, (1) owing to the responsiveness of the respiratory rate to various unavoidable sensory stimuli, and (2) owing to the fact that there is another " dimension " to the respiratory movements—depth as well as frequency. It is likely that the former may influence the amount of water passing through the gill-chambers as much as the latter, which alone can be recorded.

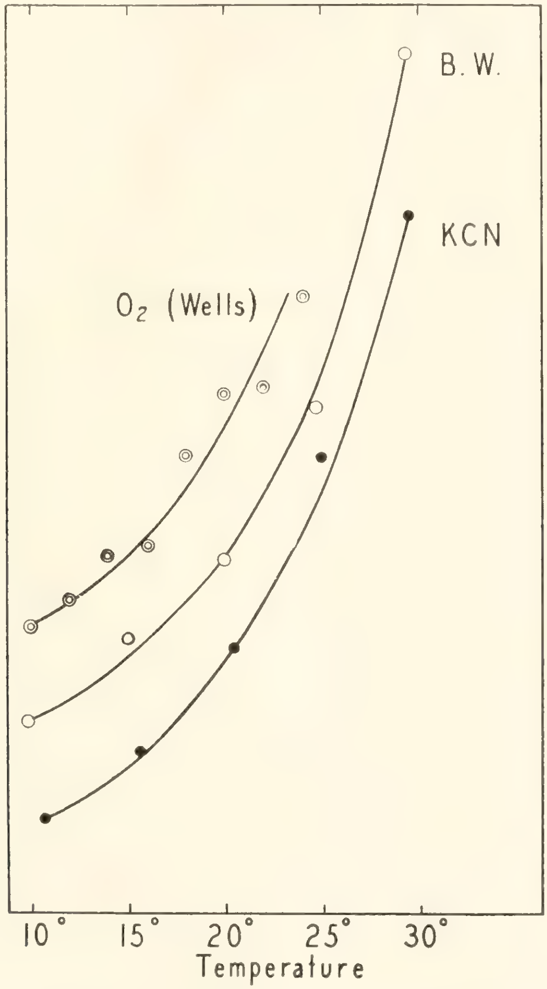

FIG. 1. Correlations between temperature and respiratory metabolism. The values for KCN and for boiled water (*B. W.*) are obtained in each case by dividing the reciprocals of the mean times of death at the various temperatures by the reciprocal of the time at 10° (equivalent to dividing mean time of death at 10° by each of the others). The upper curve is based upon measurements of oxygen consumption by N. A. Wells (1935, Table II (means of Experiments 4 and 5). Each of these curves is based upon relative values only. Their separate positions on the vertical scale have no significance.

The more significant results of all these experiments may be summarized as follows:

POTASSIUM CYANIDE

(1) Death in KCN was speedy in direct proportion to the temperature of the medium, fishes dying far more rapidly at a high temperature than at a low one. This fact is altogether in accordance with expectations, since the oxygen requirements of fishes and other poikilotherms are known to rise with increasing temperature.[4] When the reciprocals of the times of death are plotted against temperature, using the value at 10° as unity, a fairly exact logarithmic series is shown, of a form similar to that shown when the known data of oxygen consumption are similarly plotted (Table I, Fig. 1).[5] It will be seen that the Q_{10} lies between 2.6 and 2.8.

(2) A low correlation exists between the duration of acclimatization to high or low temperatures and the degree of resistance to KCN, at those temperatures. When fishes which had been subjected to 30° for a few hours, and for various numbers of days, up to a month or more, were compared in KCN at this temperature, the results were highly variable, though statistical differences of reasonable certainty were evident when all of the results were considered. From Table II it is evident that a progressive increase of resistance occurred, commencing with the briefest periods of acclimatization.

TABLE II

Relation between duration of acclimatization in 30° and resistance to KCN at the latter temperature. (Mean times in minutes).

Duration acclim.	½ hr.–5 hrs.	1 day	3 days	5 days
Number of fishes.	76	52	60	10
Length of resistance	34.67±0.25	41.79±0.42	44.52±0.53	47.90
Duration acclim.	10–11 days	22–42 days		
Number of fishes	26	42		
Length of resistance. .	50.85±0.67	48.36±0.66		

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[4] It is possible of course, that the results with KCN may be influenced to some extent by the effects of temperature on diffusion, or on the "coefficient of distribution," or some other physical factor (cf. discussion by Child, 1913), though this could hardly affect the phenomena of reversal at a common temperature.

[5] Similar curves representing the correlation between temperature and metabolism have been published by Krogh (1916), Benedict (1932) and doubtless others.

This situation may be explained on the supposition that the initial rise in respiratory metabolism, resulting from the abrupt transfer to a higher temperature, is followed by an adjustment, in which the metabolism gradually falls again (Fig. 2). That it never even approximately reaches its former level, at least within the time limits of our experiment, is obvious from an inspection of the table and graphs.

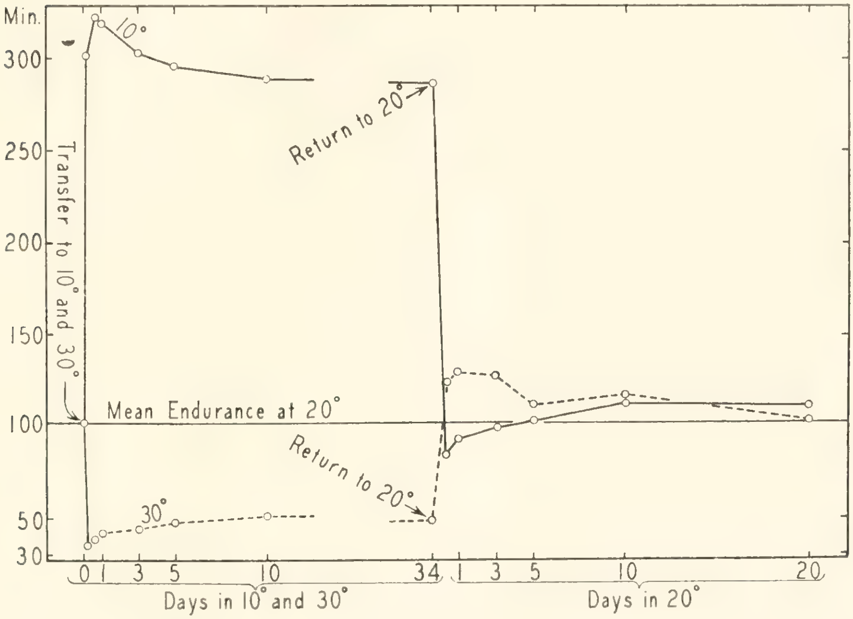

Fig. 2. Relations between duration of acclimatization in 30° and 10° respectively, and resistance to KCN at these temperatures. The 30° curve is based upon data in Table II, the 10° curve upon data in Table III. The line representing "Mean Endurance at 20°" (102 minutes) is based on all of the fishes (100) for which reliable figures at this temperature are available. Abscissas represent (left) days of acclimatization in 10° and 30° water, and (right) days following return to original temperature (20°). Ordinates represent survival time in minutes.

A similar, though converse, situation was to be expected when fishes were transferred to water of lower temperature instead of higher. An initial fall in respiratory metabolism (increase of resistance to KCN) would occur, followed by a compensatory rise, manifested in diminishing resistance. This, indeed, is what we have observed, though the variability of the individual figures is even greater than in the case of the warm water series, and the statistical probability of the successive steps is correspondingly less (Table III). A further difference between the "warm" and "cold" series is the fact that the maximum resistance of the latter (peak of the curve) is not attained as promptly as is the minimum resistance of the former, so that a further rise occurs within the period of our test, before the compensatory drop commences (Fig. 2).

(3) If, instead of subjecting the fishes to KCN, while at these temperature extremes (10° and 30°), they were removed from these last and returned to an intermediate temperature before the test with cyanide, it was found that a complete reversal took place, the fishes of "warm" history now displaying a higher resistance than those of "cold" history, while the 20° fishes remained intermediate as previously. These reversed effects persisted clearly for 3 days after transfer to the intermediate temperature, less certainly after 5 and 10 days. (Table IV, Figs. 2, 3.) The re-reversal of the relations which was found at the end of 20 days (Fig. 2) may well be accidental, as are perhaps the exact relations of both of these curves to the "intermediate" (20°) line, which is based upon the mean figure for 100 fishes.

TABLE III

Relation between duration of acclimatization in 10°, and resistance to KCN at the latter temperature. (Mean times in minutes).

Duration acclim.	½ hr.–5½ hrs.	1 day	3 days
Number of fishes	74	53	50
Length of resistance	300.8±2.92	317.8±2.86	302.8±4.32
Duration acclim.	5 days	10–11 days	24–44 days
Number of fishes	10	26	33
Length of resistance	296.3	289.1±3.81	285.5±5.58

Certain relations of more or less certain significance are to be noted from inspection of Fig. 3. Thus, the curves for the "W" (30°) series, without exception, lie above those for the "C" (10°) series; the curves of each comparable pair (equal times in "I") diverge from one another, with increasing times of acclimatization; the terminals of the curves (with the exception of "I, 2 hrs." in the "W" series) are arranged in sequence, according to the length of subjection to "I" (20°), and are arranged in reverse order in the two series. The two "I, 2 hrs." curves, for example, are farthest apart, the "I, 10 days" ones closest together, etc. The various incongruities in the figure, as a whole, are doubtless due to the high individual variability shown by each set of fishes.

(4) It is probable that within broad limits no appreciable relation exists between size and susceptibility to KCN poisoning. In two experiments, 21 "large" and 17 "small" fishes were compared, the former averaging from 4 to 5 times as heavy as the latter. The mean times of succumbing were 101.0 and 103.4 minutes, respectively, there being, in each of the experiments, an almost complete overlapping of the values.

TABLE IV

Persistence of effects of acclimatization to 10° ("C") and 30° ("W"), when followed by sojourns of varying length at intermediate temperature (20°) upon resistance to KCN at the latter temperature. Survival times in minutes. Values having high probability denoted by asterisks. For method of computing probabilities see Tippett, 1931, pp. 80–82.

Numbers in the two lots	Duration of acclimatization	Time in 20°	C	W	Difference (W – C)	Probability that difference is significant
29, 24	1 day	2 hrs.	89.7	104.4	14.7	*0.9999+
7, 10	1 "	1 day	90.7	107.2	16.5	*0.9959
10, 10	1 "	3 days	109.8	109.7	−0.1	0.5000
10, 10	1 "	10 "	100.5	101.2	0.7	0.5000
5, 5	3 days	2 hrs.	86.0	87.0	1.0	0.6500
5, 6	3 "	1 day	93.0	124.8	31.8	*0.9999+
15, 15	5 days	2 hrs.	90.5	115.5	25.0	*0.9999+
9, 10	5 "	1 day	90.8	121.3	30.5	*0.9999+
8, 10	5 "	3 days	101.4	116.0	14.6	*0.9985
10, 10	5 "	10 "	107.2	108.4	1.2	0.6300
11, 11	10–11 days	2 hrs.	83.8	107.0	23.2	*0.9999+
23, 18	33–40 d.	2 hrs.	84.2	123.2	39.0	*0.9999+
18, 20	36–51 "	1 day	92.0	129.0	37.0	*0.9999+
19, 20	36–56 "	3 days	98.5	127.0	28.5	*0.9999+
10, 10	57 days	5 "	101.7	110.9	9.2	*0.9969
18, 30	31–46 d.	10 "	111.9	116.7	4.8	0.8200
10, 10	46 days	20 "	110.7	103.6	−7.1	0.9245

BOILED SEA WATER

(1) When kept throughout the tests at the various experimental temperatures, the time relations of the deaths in boiled water were very similar to those in KCN though forming a less perfect logarithmic series (Table V, Fig. 1). The values for Q_{10} are here much less con-

stant, ranging from 2.3 to 3.0. These incongruities may be due to the considerably smaller number of individuals used.

(2) Death in deoxygenated sea water was more than 60 per cent slower, at the same temperatures, than in KCN solution. That this slower rate was not due to traces of oxygen left in our boiled water is known from the fact that considerable increases (× 2 or 3) in the small amount of oxyygen present were not found to have any appreciable effect. It may be due to the presence of a considerable reserve of oxygen in the tissues, which is rendered unavailable by the KCN. Or it may be that the latter has some toxic effect, independently of its effect upon oxygen utilization.

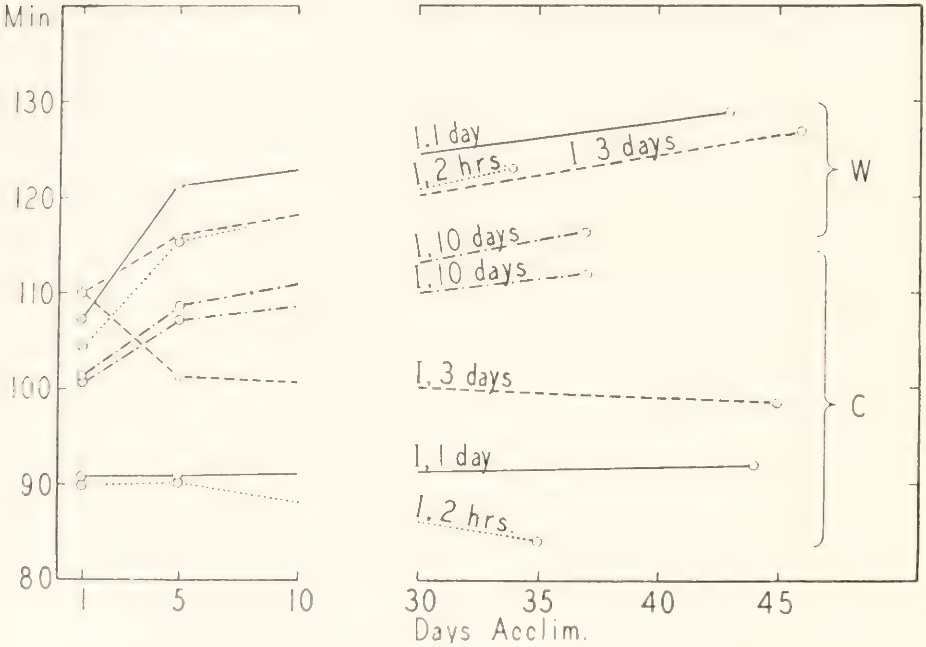

Fig. 3. Acclimatization for varying periods in "cold" (10°) and "warm" (30°), followed by sojourn for varying periods in "intermediate" temperature (20°), from which the fishes were originally taken. Abscissas = times of acclimatization in *C* and *W* (days). Ordinates = survival times in KCN solution (minutes). Separate curves are plotted for the various sets differing in their length of sojourn in *I*.

(3) Owing, in part at least, to the much smaller number of fishes used in the present series, the relations between duration of temperature conditioning and resistance to asphyxiation were not so well shown here as in the case of KCN. For those which had been acclimatized

for varying periods to 30°, a trend was nevertheless indicated, which agreed with that for KCN. This was not, however, true of the experiments at 10°.

(4) In two experiments in which "cold" lots were compared with "warm" or "control" (20°) lots, after transfer to the latter temperature, we had the same marked reversal as in the case of cyanide. Here, however, we had only 2 to 4 individuals of each sort.

(5) The size of the fishes, in experiments with boiled water, as contrasted with those in KCN or lethal heat, was found to have a marked influence upon the death-rate. Smaller (i.e. younger) fishes, as might have been expected *(for both KCN and boiled water)*, were the first to succumb. Four experiments were performed, five fishes of each size being used in each of these. In two experiments, in which the mean weight of the large fishes was 2⅓ times that of the small ones, the average duration of life of the former was 15 per cent greater than that of the latter. In two other experiments, in which the large fishes averaged 4 times as heavy as the small ones, the difference was 41 per cent. The means for the 20 large and 20 small, comprised in the entire series were: 297.4 and 238.7 minutes respectively.[6] The rapid decline of respiratory metabolism with increasing size in fishes and other cold-blooded animals is now a familiar fact. (Keys, 1931, and Wells, 1935*a*, have presented data for fishes, based upon experiments conducted in this laboratory.)

TABLE V

Mean times of survival in boiled sea water, at various temperatures, after 3 days acclimatization to these, and relative rates of metabolism on which "B.W." curve in Fig. 1 is based. For significance of figures for "relative metabolism" see Table I.

Number in each lot	Mean length (cm.)	Temperature of acclimatization (±)	Actual temperature in flasks during experiment	Times of survival		Relative Metabolism
				Mean	Range	
		°C.	°C.			
10	10.6	10	9.8	483.7	397–619	1.0
10	10.2	15	14.9	257.6	210–296	1.9
10	10.3	20	19.9	179.7	140–227	2.7
9	10.7	25	24.8	112.0	77–148	4.3
10	10.3	30	29.5	60.5	50– 77	8.0

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[6] All four experiments yielded large mean differences, while in two of these there was no overlapping of values. But the four sets are not homogeneous enough in respect to temperature, etc. to justify the computation of probabilities.

LETHAL HEAT AND COLD

(1) With fishes taken originally from a stock tank at 20°, differences in the length of conditioning to 30° led to marked differences in their subsequent resistance to lethal heat. Even a half hour's previous stay at 30° led to a considerable increase in resistance, and this increase continued, though at an ever-slowing rate, up to 10 (perhaps even 30) days, when this particular test was discontinued. After the first day, the rise of the curve was very gradual (Table VI, Figs. 4 and 5).

TABLE VI

Relations between length of acclimatization to 30° and resistance to lethal effects of heat, the resistance being indicated both by the time and the temperature at which the fishes succumbed. (Including only those experiments in which heat treatment commenced with 37.6±).

The probabilities relate to the differences between each temperature and the one in the preceding column. High probabilities indicated by asterisks.

Time of acclimatization	0	30 min.	1 hr.	2–2½ hrs.	4–5 hrs.	9 hrs.	15¾ to 19½ hrs.
Number of fishes	15	10	5	10	20	10	20
Mean time (minutes)	4.9	10.6	21.5	24.5	30.1	34.4	40.9
Mean temp.	37.73	38.03	38.36	38.48	38.73	38.89	39.06
Probabilities		*0.9999+	*0.9890	0.8740	*0.9999	*0.9940	*0.9999+

Time of acclimatization	1 day	3 days	10 days	30 days
Number of fishes	10	10	10	10
Mean time (minutes)	41.5	53.6	61.0	62.2
Mean temp.	39.11	39.32	39.62	39.68
Probabilities	0.9400	*0.9975	*0.9992	0.8300

Not included in the foregoing are three experiments in which the temperature of the "hot" tank at the outset was 38.75° (±), and in which the fishes under comparison (10 of each) had previously been conditioned to 30° for 3 and 39 days respectively. The mean lethal temperatures for the two lots were 39.50° and 40.11°, respectively, there being no overlapping of the figures for the contrasting lots.

(2) Return to a common temperature of 20° for considerable periods before testing with lethal heat diminished, but did not annul, the

effects of previous acclimatization. For example, a sojourn of 23 days at 20° did not suffice to eliminate the difference between lots which had been kept for 46 days at 10° and 30° respectively, while fishes kept for only a single day at these last temperatures retained a considerable part of the resulting difference after 10 days at 20°. On the other hand, the effects of 3 days conditioning at these extreme temperatures had nearly or quite disappeared after 30 days at the intermediate one. This last is not in agreement with certain results reported for *Fundulus* by Loeb and Wasteneys (1912), who state: "The immunity against a temperature of 35° acquired by keeping the fish for two days at 27° is not lost or weakened if the fish are afterwards kept as long as thirty-

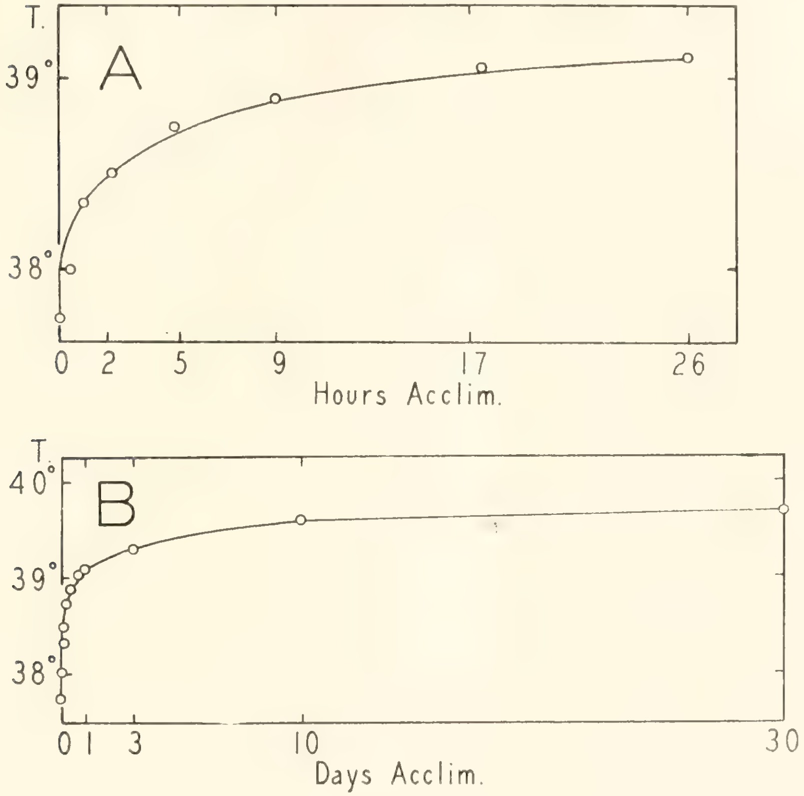

FIG. 4. Correlation between previous acclimatization in 30° tank and resistance to lethal effects of heat. Data for shorter periods only (up to one day) are plotted in *A*; those for both short and long periods are plotted, on different scale, in *B*. Abscissas = times of acclimatization in hours (or days); ordinates = mean temperatures which proved lethal to the various lots.

three days at a temperature of from 10° to 14°." It may well be that *Gillichthys* differs from *Fundulus* in this respect. These authors likewise state: "The immunity against a temperature of 35° C. is also maintained if the fish are kept after the two days' exposure to 27° for two weeks at a temperature of 0.4° C." We have not employed such a low temperature in this connection.

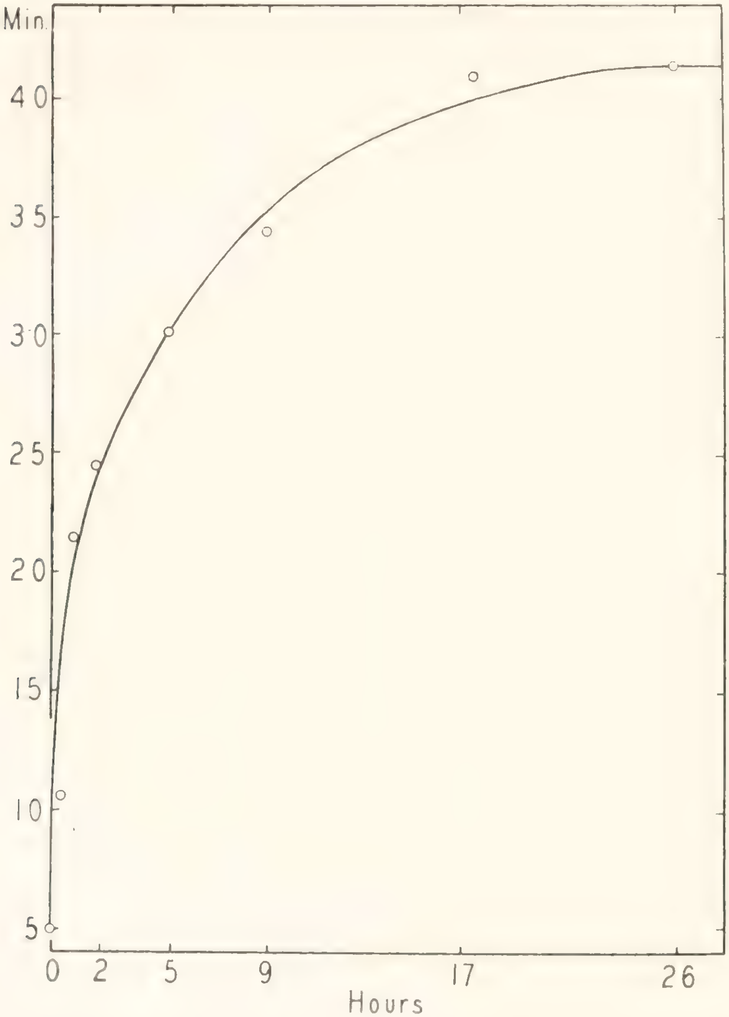

FIG. 5. This is the counterpart of Fig. 4.4, the present values representing the mean times in which death occurred, instead of the temperatures.

Table VII[7] comprises the chief results of experiments of this class, while Figs. 6 and 7 depict, for one series of tests, the relations between the periods of conditioning in "cold" and "warm" water (10° and 30°) and the subsequent sojourn at the "intermediate" temperature (20°). The divergence of the two sets of curves in Fig. 6, and their convergence in Fig. 7 represent significant facts, as do the relative positions of all of the single curves in Fig. 7 and some of those in Fig. 6.

Table VII

Persistence of effects of acclimatization to 10° ("C") and 30° ("W"), upon heat resistance, when followed by sojourns of varying length at intermediate temperature (20°).

The probabilities relate to differences between lethal temperatures for C and W lots. High probabilities indicated by asterisk.

Number of fishes in each lot	Period of acclim. (10° and 30°)	Subsequent stay at 20°	Temp. of hot tank at start	Period of resistance		Lethal Temp.		Probability
				C	W	C	W	
10 (9 in W)	6 hrs.	2(+) hrs.	36.0°	23.0	55.8	37.38°	38.56°	*0.9620
10	1 day	2¼ hrs.	36.0°	15.1	67.1	36.96°	39.09°	*0.9999+
10	" "	1 day	36.0°	28.7	61.3	37.50°	39.11°	*0.9999+
10	" "	3 days	36.0°	26.7	53.3	37.58°	38.68°	*0.9999+
10	" "	10 "	36.0°	22.2	36.3	37.31°	38.02°	*0.9997
15	3 days	30 days	36.0°	18.8	20.4	36.99°	37.07°	0.6900
10	5 days	2 hrs.	37.0°	5.0	58.3	37.30°	39.27°	*0.9999+
10	" "	1 day	36.0°	29.0	70.1	39.64°	39.53°	*0.9999+
10	" "	3 days	36.0°	13.5	64.7	36.80°	39.11°	*0.9999+
10 (9 in W)	" "	10 "	36.0°	18.9	47.4	37.25°	38.66°	*0.9999+
10	34 days	2(+) hrs.	37.0°	2.5	70.1	37.20°	39.89°	*0.9999+
10	37 "	1 day	36.0°	5.7	80.9	36.39°	40.09°	*0.9999+
10	" "	3 days	36.0°	7.4	76.3	36.54°	39.85°	*0.9999+
10	32 "	10 "	36.0°	12.7	53.7	36.82°	38.90°	*0.9999+
10	46 days	23 days	36.0°	14.1	37.6	36.87°	38.23°	*0.9999+
23‡	" "	47 "	36.0°	24.7	27.9	37.36°	37.55°	0.8850

‡ In this experiment, 10 "controls" which had been kept continuously at 20° gave mean values: time, 18.3 minutes; temperature, 37.07°.

(3) Long-time (38-day) conditioning of fishes to a low temperature (10°) made them more resistant to extreme cold (1.0° —) than

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[7] Corroboration of some of these results was obtained from a number of experiments which could not conveniently be included in the table.

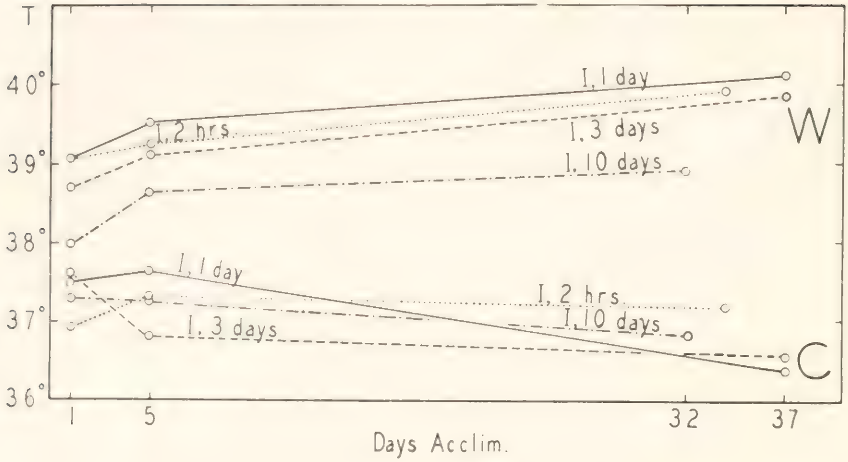

FIG. 6. Same legend as for Fig. 3, except that the present figure relates to heat resistance, the ordinates representing temperatures which proved lethal to the various lots of fishes.

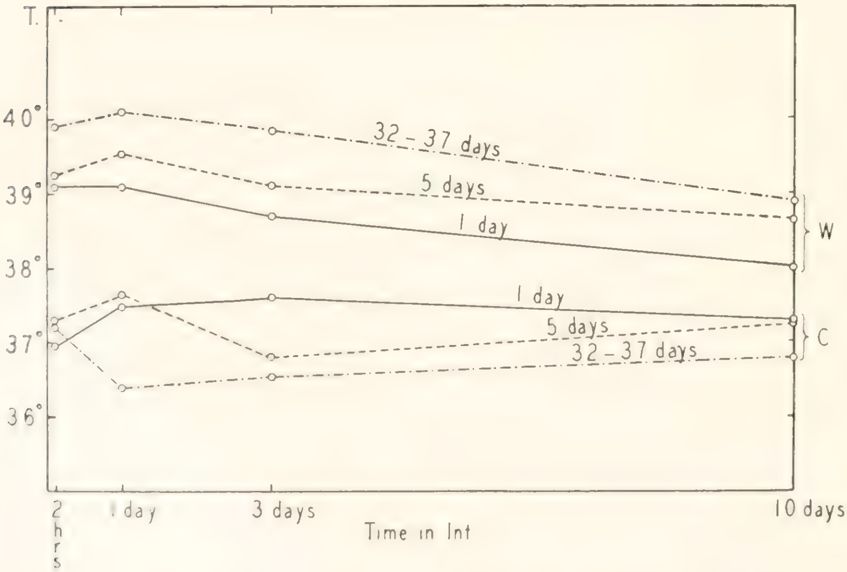

FIG. 7. Based upon the same experiments as Fig. 6, but with different treatment of the data. In the present case, the subsequent sojourn in "intermediate" (20°) is plotted on the abscissas axis, while separate curves are drawn for the various periods of previous conditioning to "cold" and "warm."

ones which were transferred to 10° only 3 days previously. Our evidence here is reasonably conclusive, though not lending itself readily to quantitative treatment (see pp. 407–8).

(4) Fishes kept alternately at 10°, 20° and 30° for 7 or 8 complete cycles were more resistant to heat than ones which had been kept continuously at 20°. This was decisively true, even in some experiments in which the "cold" periods were twice as long as the "warm" ones (Table VIII).[8] On the other hand, fishes which had been sub-

TABLE VIII

Effects upon resistance to heat of previous subjection to "cold" (10°), "intermediate" (20°) and "warm" (30°), in alternation.

Temp. of hot tank at start	Rate of rise	Number of fishes	Temperature history	Period of resistance (minutes)	Lethal temperature
37.6°–37.7°	0.1 in 13 min.	8	5 cycles: W(24 hrs.), I(12 hrs.), C(24 hrs.), I(12 hrs.), etc.	32.7	
		8	5 cycles: C(24 hrs.), I(12 hrs.), W(24 hrs.), I(12 hrs.), etc.	46.0	
		8	Control: I (continuously)	4.5	
37.0°–37.1°	0.1° in 2.8 min.	10	8 cycles: W(12 hrs.), I (12hrs.), C(12 hrs.), I(12 hrs.), etc.*	29.5	38.20°
		10	7 cycles: W(12 hrs.), I(12 hrs.), C(24 hrs.), I(12 hrs.), etc.†	42.5	38.47°
		20	Control: I (continuously)	6.0	37.30°
			Probability of difference between first and second temperature low (0.8100); between first and third very high (0.9999+)		
36.0–36.1	0.1° in 2.1 min.	10	9 cycles: W(12 hrs.), I(12 hrs.), C(24 hrs.), I(12 hrs.), etc.†	66.7	39.25°
		11	Control: I (continuously)	15.1	36.91°
			Probability of difference very high (0.9999+)		
37.6	0.1 in 2.0 min.	15	W (6 days, continuously)	42.8	39.80°
		15	W and I (12-hr. periods alternately for 12 days)	43.0	39.79°
		15	W and I (2-day periods alternately for 12 days)	43.0	39.79°

* Except that last C and W were each 24 hours.
† Thus C time = 2 × W time.

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[8] A somewhat similar experiment was performed by Loeb and Wasteneys (1912), with results similar to ours.

jected to these alternations of temperature were *less* resistant to extreme cold (0° — 1°) than ones kept continuously at 20°. This statement is based upon four experiments, involving 28 "alternation" fishes and 19 controls. While the results cannot well be treated quantitatively, for reasons already stated, this general statement admits of little doubt.

(5) When the water temperature was raised gradually, throughout a period of several hours, the tolerance was considerably increased, in comparison with experiments in which the fishes were subjected abruptly to these higher temperatures. Thus when 15 fishes were placed in a tank at 32.2° and the water gradually warmed, the first death occurred when the temperature reached 37.6°. This was 5½ hours after the commencement of the experiment, and 1¼ hours after the temperature had reached 37.2°. On the other hand, when 19 fishes of the same original stock were placed abruptly in water at stationary temperatures of 36.9° to 37.2°, they all died within a maximum of 17 minutes, the mean time being 10 minutes.

(6) In four experiments, comparisons were made of the resistance to heat of large and small individuals (totalling 21 of each), having identical temperature history. The former averaged about four times as large, by weight, as the latter. The mean temperatures at which they succumbed averaged: large, 37.79°; small, 37.48°. Comparison of individual figures makes it highly improbable that any relation exists between size and resistance to heat, or at least any which could be revealed without the use of much greater numbers of fishes.[9] This is in marked contrast to the consistent differences which were generally to be observed in comparing fishes of different temperature history.

(7) In a single experiment, the question was tested whether resistance to heat was affected by the salt concentration of the water. Unlike Loeb and Wasteneys (1912), we used for this test a fresh water species (*Gambusia affinis*). This fish can be acclimatized to 75 per cent sea water (probably to full strength) if the change is made in several steps. In our single experiment, two lots, of 17 and 16 fishes respectively, which had been kept in fresh water and 75 per cent sea water (the latter in this medium for 5 days), were subjected to lethal heat. The mean temperature of succumbing of the fresh water individuals was 37.46, that of the salt water ones 38.53. If one individual were removed from each series, there would be no overlapping between the two. Thus heat was more rapidly fatal to this fresh water species

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[9] Belehradek (1935) cites contradictory figures as regards the relation of age to heat-resistance in fishes. M. M. Wells (1914), on the other hand, on the basis of experiments upon several species of fresh-water fishes, concludes: "Large fish of a given species are more resistant to high temperatures than small fish of the same species." Wells used *Ameiurus melas* and several species of *Notropis*.

in fresh water, than in the unfamiliar medium to which it had been rather rapidly acclimatized.

URETHANE

(1) In comparing the times required for anesthetizing at different temperatures, no consistent results were obtained. The earlier inference (Sumner and Wells, 1935) that the effects of urethane were directly related to the rate of respiratory metabolism does not appear to be well founded.

(2) Nevertheless, when acclimatization at 10° and 30° was followed by sojourn at a common temperature (20°), tests with urethane, made at the latter temperature, gave consistent results similar to those reported by Sumner and Wells, and agreeing with those reported in the present paper for KCN and boiled water. Fishes of previous " warm " history were much more resistant than ones of previous " cold " history, and these differences tended to be great in proportion to the duration of the previous acclimatization.

(3) When comparison was made of fishes (10 each) which had been kept at 30° for 5 hrs., 1 day, 3 days and 34–35 days, a consistent increase in resistance was manifested, the last set requiring nearly three times as long for anesthetization as the first.

(4) No consistent results were obtained when similar tests were made at 10°.

This last fact is due, in part, to the difficulty in finding a satisfactory " end-point " for urethane anesthesia at low temperatures. For this and other reasons, we abandoned the plan to make more extensive tests with this drug. While the results stated in paragraphs 2 and 3 of this section appear to represent actual physiological differences, we are not disposed to attempt any interpretation of these at present.

RESPIRATORY MOVEMENTS

(1) The frequency of respiratory movements, based upon 45 " cold " (10°) fishes, 78 " intermediate "[10] (20°) ones, and 44 " warm " ones, was 26.4 ± 0.61, 73.7 ± 1.15, and 74.8 ± 1.95, respectively, per minute. The figures upon which these averages are based are restricted to experiments in which fishes at all three temperatures were available for simultaneous comparison. It is unlikely that the " warm " and " intermediate " fishes differed significantly (Table IX).

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[10] This number included many of the " warm " and " cold " fishes, which were tested at 20°, before subjection to the other temperatures.

(2) Transfer from 20° to 10° resulted in a mean fall in respiratory rate of 39.7 ± 2.02 per minute (mean of 39 fishes). After return to 20°, this was followed, in the 37 fishes thus tested,[11] by a mean rise of 32.5 ± 1.34 per minute.

(3) Transfer from 20° to 30° resulted, quite unexpectedly, in no consistent rise in respiratory rate. In seven experiments (3 fishes each), in which counts were made of the breathing of fishes that had been transferred to the 30° tank from 2 hours to 4 days previously, the mean rise in respiratory rate was a nominal one (1 per minute), there being actually more negative cases than positive ones. On the other hand, there was a large and consistent *fall* when 30° fishes were returned to 20°, the average difference for 28 fishes being 33.3 ± 2.50 per minute. These two sets of facts are difficult to reconcile with one another.

TABLE IX

Mean rates of respiratory movements at 10° ("C"), 20° ("I") and 30° ("W")

Duration acclim. in C and W	Temp. history	Number of fishes	Mean length (cm.)	Resp. per minute
3–4 hrs.	C	6	12.7	36.0
	I	18	12.7	73.5
	W	6	12.8	86.7
18–24 hrs.	C	12	13.1	27.5
	I	30	13.0	73.2
	W	12	13.3	70.8
2 days	C	9	13.1	26.1
	I	24	13.0	72.6
	W	8	12.7	74.8
10–11 days	C	12	13.2	22.4
	I	12	13.0	76.6
	W	12	13.2	81.2
33–37 days	C	12	13.7	29.4
	I	12	14.3	74.4
	W	12	13.9	72.5
Means (omitting 3–4 hours)	C	45	13.29	26.4±0.61
	I	78	13.20	73.7±1.15
	W	44	13.33	74.8±1.95

(4) When fishes of "warm" and "cold" history (28 of each)

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[11] Mostly different individuals from those covered by the statement immediately preceding.

were compared 15 minutes to 2 hours after transfer to 20°, the latter gave a distinctly higher rate, this difference averaging 15.9 ± 2.73 per minute. Here we had the same reversal of relation as was found with KCN and boiled water.

(5) The rate of respiratory movements was dependent to some extent on size. In each of 4 experiments, 5 " small " and 5 " large " fishes were used, the temperatures being 15.1° to 15.5°. The 20 small ones, having a mean weight of about 34 grams, gave a mean rate of 49.1 respirations per minute, as compared with 26.9, for the 20 large fishes, which averaged about 73 grams. In these four experiments, only a single case of overlapping occurred between the two contrasted lots.

Discussion

From the various quantitative relations revealed in our tables and curves, it is conceivable that definite conclusions might be drawn regarding the nature of the various chemical reactions concerned. The present writers do not feel disposed, or indeed qualified, to undertake any such general interpretations of our results. It may, however, be instructive to point out analogies between certain features of these results and some other biological phenomena.

The situation depicted in Fig. 2 seems to find an instructive counterpart in the field of sensory physiology. The intensity of a sensation rises rather abruptly to a maximum, following a stimulus, and then falls again, even while this stimulus is continued. The crossing of the two curves in our figure, following the return of the two sets of fishes to a common, intermediate temperature, recalls an old and familiar experiment in sense physiology. We refer to the one in which the subject's two hands are dipped into hot and cold water, respectively, and then, after a few minutes, they are removed and dipped simultaneously into water of medium temperature. One experiences a reversal of his temperature perceptions quite parallel to the reversal in metabolic rate which is manifested by the fishes after a similar transfer. Such an analogy is not, of course, to be offered as a substitute for an explanation in physico-chemical terms. But in the absence of a satisfactory explanation of the latter type, it may have suggestive value. Indeed, it seems likely that this analogy is based upon fundamental similarities in the irritability of quite different sorts of living matter.

The relations shown in Fig. 1 are representative of a wide range of phenomena which have to do with the effects of temperature upon the rates of vital processes. (Cf. Krogh, 1916; Bělehrádek, 1935.) The vast and well-tilled field of " temperature coefficients " (cf. Bělehrádek, 1935, Heilbrunn, 1937) is one which we have no intention to discuss.

Our curves (Figs. 4 and 5), are quite definite in their trend, though we doubt whether they furnish anything very distinctive by which the underlying chemical or physical processes could be identified. Perhaps others might find this possible. It hardly seems likely, however, that such a rapid acclimatization to heat could result from the production of fats having successively higher melting-point (Heilbrunn, 1937; Bělehrádek, 1935), though we should not be warranted in denying this possibility. Biochemical processes in the field of sense physiology are known which are even more rapid than this, e.g. light- and dark-adaptation in the human eye (Hecht, 1920, Tansley, 1931). The striking similarity between our curves (Figs. 4 and 5) and some of those offered by the latter writer perhaps deserves mention. Tansley calls attention to the close parallel between the rate of a sensory change, determined subjectively, and a measured biochemical change (restoration of visual purple), which is presumed to be intimately associated with this. Some future physiologist may be able to prove that heat-acclimatization rests upon an equally simple chemical transformation.

Regarding the much-discussed question of the cause of death at (moderately) high temperatures, we can make no contribution of a positive nature. We can, however, offer reasons for doubting the adequacy of one of the agencies which have been proposed, namely a fatal rise in the rate of respiratory metabolism. We have already called attention to the difference between the effect of temperature conditioning upon resistance to heat and its effect upon resistance to asphyxiation by boiled water or by cyanide.

The former effect was of far greater magnitude than the latter. Resistance to heat, as measured by survival time, increased eight-fold in the course of one day, and continued to increase quite appreciably for some days further. Resistance to KCN, measured in the same way, increased about 50 per cent in the first day, and any further increase was only detectable statistically. Increase of resistance to asphyxiation in boiled water was even less obvious than in the case of KCN, though this last fact may have been due to the smaller numbers of fishes which we employed.

There were equally striking differences in the *persistence* of the effects of heat-conditioning upon resistance to heat and to asphyxiation. The effect upon heat resistance was very much more lasting, after withdrawal of the original temperature conditions, than was the effect upon resistance to KCN. (Boiled water was not tested in this connection.)

Still another difference of interest, already mentioned, concerned the relation of size to susceptibility to these lethal agents. As regards heat resistance, no difference was found, within rather broad limits. In boiled water, on the other hand, the smaller fishes rather uniformly died earlier, as might have been expected. Quite unexpectedly, however, no such difference was found in experiments with KCN. For this we can offer no present explanation.

It would appear, therefore, that there are at least two distinct processes involved in acclimatization to a higher temperature: (1) a regulative decline in the initially much increased respiratory metabolism; and (2) another change of an unknown nature (either physical or chemical), by which the threshold of susceptibility to destructive temperature is raised.

SUMMARY

Fishes (*Gillichthys mirabilis*) having different temperature histories were subjected to KCN, to boiled sea water, and to temperatures high or low enough to be lethal. Urethane, an anesthetic, was used in a limited number of experiments. The time of death (or of anesthetization) was recorded for each of the fishes (nearly 3,000). Counts of respiration were made in a considerable number of cases.

Death in both KCN and in boiled water was speedy in proportion to the temperature, the reciprocals of the times of death forming an approximate logarithmic series when plotted against temperature. The temperatures used were 10°, 15°, 20°, 25° and 30°.

In the case of KCN, a low correlation was found to exist between the duration of previous acclimatization to high or low temperatures and the degree of resistance to KCN at those temperatures. The abrupt decrease of resistance (increase in metabolic rate), resulting from transfer to a higher temperature, was followed by a slight increase of resistance which continued for several days. On the other hand, the abrupt increase of resistance (decrease in metabolic rate), resulting from transfer to a lower temperature, was followed by a slight decrease which continued for several days.

In boiled water, this compensatory trend was only shown after transfer to higher temperatures.

When fishes which had been acclimatized to high (30°) and low (10°) temperatures were tested in KCN, boiled water or urethane at 20°, there was a consistent reversal in the resistance of the respective lots, the 30° fishes now being much more resistant than the 10° ones. These relations continued for several days after transfer to the intermediate temperature.

Death in de-oxygenated sea water was more than 60 per cent slower, at the same temperatures, than in KCN solution.

Acclimatization at 30°, even for brief periods, resulted in a marked resistance to the lethal effects of heat. This result was already conspicuous, after a half hour at 30°, and increased, at a diminishing rate up to 10 days or more.

Return to a common temperature of fishes having "cold" and "warm" histories diminished, but did not annul, the effects of previous acclimatization upon heat resistance. Pronounced differences were noted in one experiment, after 23 days at the intermediate temperature. Interesting relations were pointed out between the period of conditioning and the persistence of the effects.

Acclimatization at 10° increased resistance to lethal cold (1.0°—).

Fishes kept for alternating periods at 10°, 20°, and 30° (even when the 10° periods were twice as long as the 30° ones) were more resistant to lethal heat than fishes kept continuously at 20°. Such fishes were, however, less resistant to lethal cold than the controls.

The rate of the visible respiratory movements was much greater at 20° and 30° than at 10°. There was no certain difference, however, between the rates at the first two temperatures.

Transfer of fishes of "cold" and "warm" history to an intermediate temperature resulted in the same reversal in relative respiratory rates as was found in the case of resistance to KCN, etc.

The size of the fish appears to have little or no influence upon its resistance to lethal heat or to cyanide poisoning. On the other hand, it was found that smaller fishes had a more rapid respiratory rhythm, and succumbed more rapidly in boiled water than larger ones.

Acclimatization to higher or lower temperatures brings about two classes of effects which seem to be largely distinct from one another. One of these concerns increased resistance to the harmful effects of heat and cold, when these are used as lethal agents. The second of these concerns changes in respiratory metabolism, and therefore in resistance to oxygen lack, including KCN poisoning. Reasons are given for believing that the former effect is not dependent upon the latter.

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INTERMEDIATE-WINGED APHIDS AND THE TIME-OF-DETERMINATION THEORY[1]

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Transplantation experiments by Spemann (1927), Mangold (1928), and many others have shown that the destinies of different parts of vertebrate embryos are fixed at different times in the embryogeny. The same technique is not readily open to the investigator of invertebrate development, but it has been assumed that there is a similar succession of embryonic segregations in these groups. One important genetic theory, that of Goldschmidt (1931), concerning the nature of intersexes, states that they begin development in the manner characteristic of one sex, that a physiological change constituting a turning point occurs later, and that subsequent embryogeny is characteristic of the other sex. An organ which is determined before the turning point is reached will be like that of the former sex; one which is determined after the turning point will be like that of the latter sex. An individual produced as a result of the change postulated above is a time-mosaic of male parts and female parts whose embryonic segregations occur at different times in development. This concept of development has also been applied to intermediates in general and has been termed the time-of-determination theory (Shull, 1930). Since intermediates may conceivably be produced without any time intervals between the determination of their different structures, it is desirable to discover whether such a succession of events does occur.

Intermediates of such a nature that they may be used to test the applicability of the time-of-determination theory are not common. But when in a species of organism there exist two forms of individuals built on the same general structural plan, but differing with respect to recognizably homologous parts, definite information regarding the time of determination of the differential parts can be gained, provided each of these forms can be converted by stages into the other. Previous to Shull's (1930) work on aphids, efforts to obtain this information have dealt almost exclusively with intersexes which are the product of bisexual reproduction. The genetic composition of such intersexes is presumably variable. Intermediate aphids parthenogenetically produced have the advantage of allowing only a minimum of opportunity for the embryonic process to be influenced by genetical variation, and therefore insuring that genetic and developmental factors will not be confused in the results.

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[1] Contribution from the Department of Zoölogy, University of Michigan.

The test of serial embryonic segregation (embryonic determination) lies in obtaining intermediates by an approach from opposite extremes. Shull made such a test on aphids which could be changed from parthenogenetic to gamic, and from gamic to parthenogenetic. This change from one form to the other results in the production of many aphids which are structurally intermediate between the gamic and parthenogenetic. If the time-of-determination theory be applicable to the intermediates produced, the order in which the structures change should be the same in both; but, since in one of the changes a given organ should appear, while in the opposite change that organ should be lost, the combination of characters in the intermediates produced should be quite different in the two cases. In fact, they should be complementary in the complex of organs concerned.

In Shull's early experiments the combination of characters in the aphids obtained by this method were not complementary; the order of the change was apparently reversed, while the development of the intermediate characters was identical regardless of the direction of change. To explain these results which appeared to oppose the time-of-determination theory, Shull discusses the possibility of a physiological level hypothesis as an alternative. According to the physiological level theory, the degree of intermediacy might depend upon the state in which some physiological property existed at some critical time. If all the differentiating structures responded equally to changes in the level of this physiological process, the question of the order of determination would not arise. All organs would appear to be segregated at the same time, or during the same part of the embryogeny. Since, however, one structure might respond to a small increase of a hormone, for example, so that it became changed, while another less susceptible does not, there develops a situation in which a difference in the time of determination of these structures may be suspected, although such a difference were non-existent. This explanation is opposed to the theory that the time of embryonic segregation determines the combination of characters in the intermediate individuals. Shull's (1933) final study of a heterogeneous group of intermediate aphids of the gamic-parthenogenetic types showed that about three-fourths fell into categories to which the time-of-determination theory was applicable, and nearly one-fourth showed irregularities which could not be made to harmonize with this theory, or which required shifts in the order of determination of the several organs studied.

Shull (1937) reports a different type of intermediacy, one in which the intermediates are structurally between the winged and wingless parthenogenetic forms. The intermediates reported in the present paper are of the winged-wingless type. In fact, the intermediates discussed here were a part of the 9,152 reported by Shull (1937).

Shull (1937), after discussing many different possible hypotheses which might account for intermediate-winged aphids, states that whether intermediates are in any way dependent on the *direction* of some physiological change could not be judged from the experimental methods of producing them. Then he adds that analysis of the characters of intermediates will be needed to answer this question. This investigation represents an effort to make a more thorough study than one involving only external characters to which Shull (1937) confined his studies. There are many limitations to this method, for the possible internal development of an organ before there is any external evidence may be of great importance in explaining the developmental basis for intermediate-winged aphids.

The structures involved in intermediate-winged aphids are more minute than in the gamic-parthenogenetic intermediates (Shull, 1930) which were examined internally by gross dissection. Accordingly, the winged-wingless intermediate aphids of this investigation, after their external organs were rated, were prepared for histological and cytological studies of the internal structures, thus making possible a thorough analysis of the whole intermediate complex.

The analysis of the intermediates so studied provides the subject matter of this paper, which records attempts to test not only the relative merits of the time-of-determination and physiological level theories as applied to intermediate winged aphids, but to determine the order of embryonic segregation if the time-of-determination theory proved applicable; and to consider other related developmental phenomena. The author wishes to express here his great indebtedness to Processor A. Franklin Shull under whose direction this work was done.

THE STOCKS OF INTERMEDIATES

All aphids used in this study were from stocks which Professor A. Franklin Shull had been keeping for several years under laboratory conditions. They were from two different strains of the species *Macrosiphum solanifolii*, which have been designated the 1923 and 1931 (Shull, 1937) stocks. Shull (1937) describes in detail the experimental methods used in the production of winged-wingless parthenogenetic female aphids.

External and Internal Differentiating Characters

A complete description of the external characters of typical winged and wingless forms of *Macrosiphum solanifolii* has been given by Patch (1915). The winged female differs from the wingless in possessing wings, three ocelli on the head, wing muscles in the thorax, extra sensoria on the third segment of the antennæ (4–6 in the wingless, 15–18 in the winged), and dark color in the third segment of the antennæ. The first three organs named proved of the most critical value for this study.

Internal differences between the winged and wingless are, for the most part, correlated in some way with the external distinguishing characters. The wing muscles of the winged individuals are striated muscles, both vertical and horizontal in position. The protocerebral lobes of the brain to which the ocellar nerves are connected in the winged type show greater development than in the wingless forms. No qualitative or quantitative differences in the " symbiotic organ " were observed. There is a practical difficulty involved in making quantitative comparisons as the number of " symbionts " gradually decreases with the age of an aphid (Uichanco, 1924).

Technique

Toto mounts of intermediate-winged aphids were of some value in comparing the relative development of the median ocellus to the lateral ocelli. Of a variety of methods used for this toto mount work, the best proved to be fixation in Bouin's fluid, dehydration with ethyl alcohol, clearing with xylol and mounting in dammar.

Histological and cytological studies which involve quantitative considerations necessitate a technique which results in almost faultless preparations. If the tissue is to be satisfactory there must be no tearing, shattering or distortion. A great many techniques with many variations were tried in an effort to obtain the most satisfactory method. None of the techniques proved satisfactory until the n-butyl method for animal tissues described by Stiles (1934) was used for dehydration and infiltration. Legs, wings, and antennæ were snipped off with DeWecker eye scissors to facilitate handling and to improve sectioning of the aphids. The sections were cut 7 micra and stained with Ehrlich's acid hematoxylin as a nuclear stain and erythrosin for the cytoplasm.

Description of Intermediates

General Anatomy

A diagnosis of intermediacy of the winged-wingless aphids has generally been based on external features and as a result intermediates of this type have been defined as aphids that have started to develop wings but have failed to complete such development. That this is not by itself a diagnostic feature of intermediacy is shown by the fact that there is histological evidence of internal intermediacy without external wings. Nevertheless, the investigation described in this paper is limited almost exclusively to intermediates which could be recognized by their wing characters, because of the enormous amount of labor involved in making histological preparations to identify intermediacy from the internal organs.

The wing character in intermediates is very variable. The partially developed wings in an intermediate may vary in development from a barely perceptible roughness in the thoracic region to .95 of normal development, the latter type being very unusual. Drooping wings was a character common to all the seven species of winged intermediates described by Baker and Turner (1916), and they considered this due to a lack of supporting muscles. While many wings examined by the writer were badly crumpled and misshapen, there was no decided tendency to droop; and even if there had been, it is doubtful if it could have been explained on the basis of a lack of muscle development as many intermediates have muscles well developed in proportion to the amount of wing. Unusual cases were those in which the wings were about one-half developed and would have shown muscle through the cuticula of the dorsal thorax but did not because of a degeneration (to be discussed later) which destroyed the muscle. Fat globules could be seen through the cuticula where muscles would normally have been seen.

Antennal color may be like that of the winged or wingless, or it may be some grade between these extremes (Tables I to IX). The sensoria, although variable, are often no greater in number than in the wingless,

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EXPLANATION OF PLATE I

Fig. 1. Photomicrograph of a transverse section of the head of aphid 1103 d (4), showing on the left side considerable development internally of the ocellus before there is any thickening of the cuticula to form a lens (× 134).

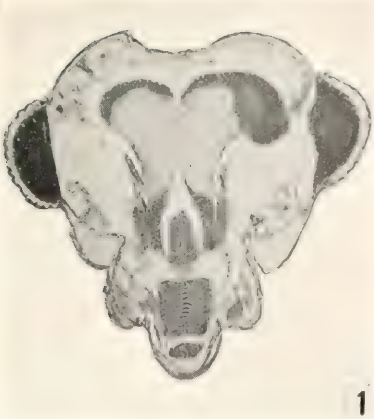
Fig. 2. Photomicrograph of a transverse section of the mesothorax of P W 48 (1), showing the wing muscle of a fully-winged aphid (× 105).

Fig. 3. Photomicrograph of a transverse section of the mesothorax of aphid 1087 g (2), showing the reduced wing muscle of an intermediate (× 117).

Fig. 4. Photomicrograph of a transverse section of the mesothorax of P 41 (1), a fourth instar winged aphid (× 88).

Fig. 5. Photomicrograph of a transverse section of the mesothorax of P 25 (2), a fourth instar intermediate aphid showing degenerating wing muscle (× 95).

Fig. 6. A photomicrograph taken with oil immersion lenses to show the detail of degenerating wing muscle of a fourth instar aphid, P 25 (3). The light-colored tissue in the upper part of the picture is normal muscle. Note especially the dark spherical degenerating cells between the muscle masses (× 917).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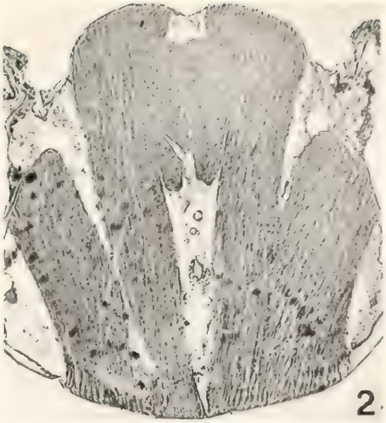
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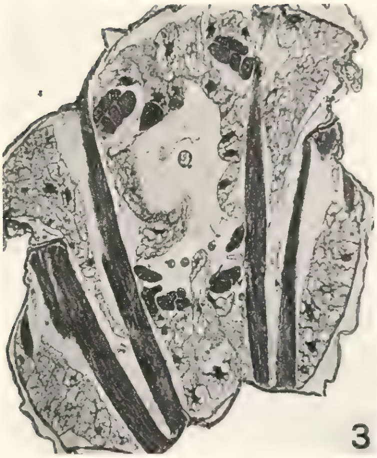
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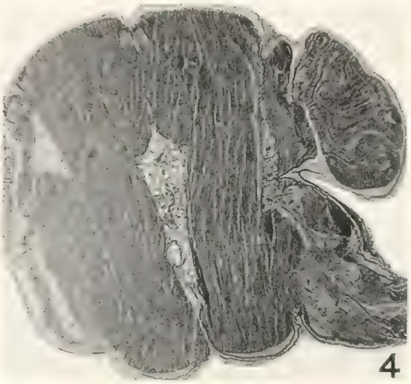
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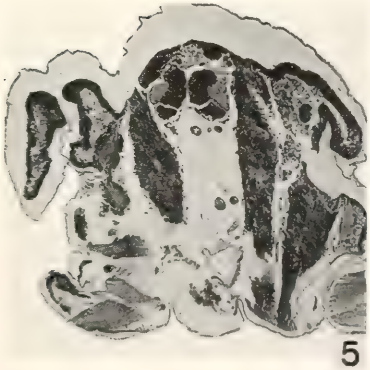

5

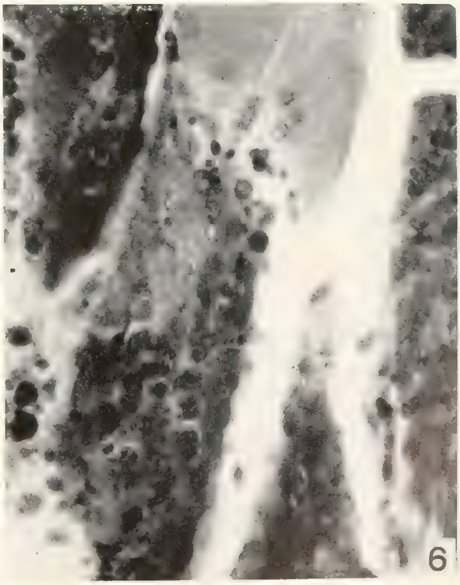

6

PLATE I

and uncommonly approach the high degree of development in the winged (Tables I to IX).

Rudimentary ocelli are frequently present in various degrees of formation. There are many cases in which no ocelli can be seen externally with either a dissection or compound microscope, but histological preparations gave positive evidence of a partly formed structure internally (Fig. 1).

The histological structure of the wing muscle in intermediate aphids may be characteristic of that in the typical winged or it may be degenerate in varying degrees. Wing muscles (Fig. 2) which in the typical aphid occupy so much of the space in the thorax, in the intermediate (Fig. 3) are usually less extensive. Degeneration of larval muscles resulting from histolysis in insects with a complete metamorphosis is well known. But in groups with a paurometabolous (incomplete) metamorphosis there is usually a continuous transformation of the internal organs from the immature stages to the adult without histolysis of muscle or other internal structures. With these well-established facts in mind, one would not expect to find degenerating wing muscles in intermediate aphids unless it developed that they possessed marked physiological differences from normal types. However, nearly one-half of the intermediates studied showed wing muscle with some degree of degeneration. This degeneration expresses itself in conditions which range from almost complete dissolution of the wing muscle to muscle that can be told from normal only with difficulty. The histology of this degenerating wing muscle is in some respects unlike that which has been described for the histolysis of the wing muscle of the queen ant, *Lasius niger,* by Janet (1907). The degeneration in the ant wing muscle takes place soon after the nuptial flight. At the beginning of histolysis, striations may be seen in the muscle but they become faint and finally disappear. Then the sarcostyles undergo disintegration but the nuclei persist for some time. The story of histolysis in wing muscles of the intermediate-winged aphid differs most from that of the ant in that embryonic muscles are involved.

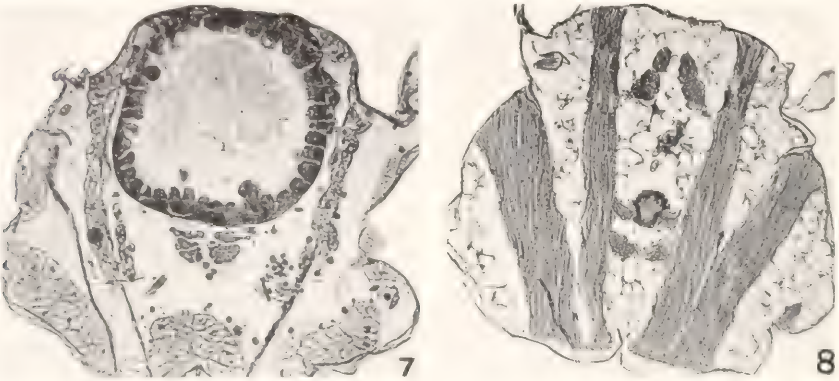
The earliest stages in which the histolysis has been observed in the thoracic muscle of intermediate-winged aphids is the fourth instar. At this stage in the normal metamorphosis the wing muscle has about completed its development and the muscle generally exhibits the character of that of the imago.

Comparison of a fourth instar with normal muscle (Fig. 4) and a fourth instar which shows degenerating muscle (Fig. 5) will indicate some of the general differences to be found. The intermediate nature of the muscle in Fig. 5 is indicated by the small amount. Both aphids were prepared by a similar technic and stained with Ehrlich's hematoxylin. The degenerate muscle in Fig. 5 is distinctly basophilic whereas the normal muscle in Fig. 4 is decidedly eosinophilic. This basophilic nature of muscle is characteristic of the periphery of the developing muscle mass of the earlier instars. This might suggest embryonic rather than degenerating muscle, but in normal developing muscle, while the periphery of a muscle where mitoses are taking place most rapidly may be basophilic, the median portion which has earlier become differentiated is eosinophilic. So in embryonic muscle the differentiation is from the center of the muscle mass to the periphery. There are, however, instances in which the degeneration of muscle is slight and these simulate somewhat the developing wing muscle of early instars.

Peripheral degeneration is interpreted as a result of the greater susceptibility to environmental influences which embryonic cells may show. Mottram (1913) with radiation has shown that *Ascaris* eggs are eight times as susceptible during division as during the resting stage. That is, there are eight times as many deaths following an exposure made during mitosis as during the later period. It has also been observed that cancer tissue is much more susceptible to radiation when it is growing rapidly than when it is nearly stationary. The last part of the aphid wing muscle to remain embryonic is the periphery and if degeneration of wing muscles, as the author believes, is due to an environmental influence, the peripheral tissue would be most affected. Figure 6 is a high power photomicrograph of the wing muscle in a fourth instar intermediate-winged aphid showing something of the more detailed structure of unusually degenerate muscles. The very dark spherical cells, lacking uniformity in size, which are to be seen in the spaces between the masses of muscles, are in advanced stages of cell degeneration. These cells in the advanced stages of degeneration seen in intramuscular spaces were doubtless at the periphery of the muscles shown and were least developed when the degenerating influence became effective. It may well be that they were more susceptible because of their extremely embryonic character. As is seen, these cells stain intensely with hematoxylin. The small amount of light colored muscle in the upper part of the picture is normal tissue which had reached the definitive stage and was unaffected by whatever influence caused the embryonic cells to degenerate. It was noted that not only mature wing muscles, but other definitive muscles of the body do not seem to suffer degeneration even though there may be marked histolysis of the embryonic muscles present.

Figure 6, in which histolysis is most active, is favorable material for studying some of the details of the process of degeneration. A looseness of the muscle tissue is apparent, distinct outlines of muscle cells may be seen which normally would be so closely crowded together that they could not be delimited from one another. In a given mass of degenerating muscle the tissue least affected is that farthest from the periphery.

Adult intermediate-winged aphids do not often show as much degeneration of wing muscles as is illustrated by Fig. 7. The wing muscles in Fig. 7 have suffered degeneration to the point where only unstriated loosely fibrous strands of tissue crowded with irregular, densely staining pyknotic nuclei remain. Bardeen (1900) points out that nuclei with a strong affinity for stain are characteristic of degenerating muscle in the pig embryo. Associated with the degenerate vertical muscle on the right side may be seen a group of either degenerate muscle cells or phagocytes distended with muscle debris. Some of the larger of these spherical bodies are muscle cells undergoing dissolution, but some of the smaller have the characteristics of leucocytes which are filled with digesting muscle fragments.



FIGS. 7–8

FIG. 7. Photomicrograph of a transverse section of the mesothorax of aphid 220 e, an intermediate with .6 wing development. The vertical wing muscles are loose, fibrous, unstriated strands of tissue, an example of extreme muscle degeneration in the adult (× 115).

FIG. 8. Photomicrograph of a transverse section of the mesothorax of aphid 1063 g (2), with .22 wing development and histologically normal wing muscle (× 128).

The longitudinal as well as the vertical muscles are involved in the degeneration process. The longitudinal muscles have so completely degenerated in Fig. 7 that there are only a few degenerate fragments left. The question whether a wing muscle degenerates or not depends, as has been pointed out, chiefly on its stage of development when conditions for degeneration obtain. The importance of wing muscle degeneration in intermediate-winged aphids in its relation to the time of embryonic determination of organs will be considered in another part of this paper.

It would be logical to examine the developing wings and ocelli in nymphal winged intermediate aphids with degenerating muscles to see if they experience any histolysis. If reference is again made to Fig. 5, the wing will be found to be reduced in size for a fourth instar and there are indications of degeneration. Cells of hypodermis of the lower and upper side of the wing, instead of being elongated as in the normal developing wing, tend to become spherical in some instances, and show a marked affinity for hematoxylin as do the degenerating muscle cells. Other disintegrating cells do not become spherical, but the cytoplasmic part of the cell can plainly be seen to be breaking down. The wing tissue in process of degeneration exhibits a marked affinity for hematoxylin. Degenerate cells are scattered in the space between the upper and lower sides of the wing bud.

From this study of the internal microscopic anatomy of intermediate aphids it is seen that there are two distinct anatomical situations, one in which the organs concerned with intermediacy are normal in histology, the other in which at least the wing muscle and developing wing bud show more or less degeneration.

Measures of Intermediacy

If intermediate aphids owe their intermediacy to a change in embryonic development from that characteristic of one normal type to that of the other type (winged to wingless or wingless to winged), and if the differentiating structures are determined at different times, it must be expected that these structures would attain different grades of advancement in different individuals. These grades of advancement are, indeed, the criteria by which the time-of-determination theory must be judged. It is necessary, therefore, to have some means of recording their degrees of development. Objective methods of measuring structures were employed when practicable. To the other characters numerical values were arbitrarily given, 0 representing the extreme found in one form, and 4 the opposite extreme in the other type. This classification of the external characters was done on living animals which had been etherized. A binocular dissection microscope usually gave magnification enough for external examination, although there were times when a question arose concerning the presence of an ocellus primordium, and this would be decided with the aid of a compound microscope.

With respect to ocelli, 0 designates a complete absence of ocelli, which is typical for the wingless viviparous females; 4, the fully developed ocelli of a winged individual; and values between these two extremes represent varying degrees of intermediacy. For example, 1 would mean ocelli one-fourth developed.

The light color of the antennae in the wingless female was rated 0, and the dark color of the winged as 4, with intergrades between these two numbers of 1, 2, and 3. An actual count of sensoria on each antenna was made; and while there was no effort to distinguish between right and left, each was tabulated separately.

Wings were used in this study as a standard of intermediacy for rough classification; that is, reference to degrees of intermediacy without regard to internal structures was based on the amount of wing development. When an aphid is said to possess a low degree of intermediacy, it means that there is little wing development although the wing muscles might be well developed; and conversely, when a high degree of intermediacy is designated, it means that the wing development is relatively great irrespective of other structures concerned in intermediacy. The wing was chosen as a standard of reference because it is an external differential character which can most easily be rated. Wings were rated from 1 to .01, the former number meaning that an aphid was fully winged, and the latter that the wing was .01 of the normal length. Some wings were less than .01 of the length of normal wings; in fact, there was a fair-sized group in which the wings were represented only by a roughness of the thoracic region where wing buds should appear. These in the raw data were termed subintermediates, to indicate a very low degree of intermediacy. These subintermediates in Tables I to IX, are indicated by a negative sign (—) after .01, as .01 —.

The degree of internal intermediacy was judged by quantitative means. For this work transverse serial sections were found most suitable. Measurements of all the internal differential organs were taken with an ocular micrometer. In rating the ocelli, the diameter was first measured and then the number of sections counted.

The wing musculature in transverse sections (Fig. 2) consists of two inner and two outer vertical muscles, together with what appears to be a single rectangular mass of longitudinal muscles divided by a median fissure. Only the maximum width of the inner and outer vertical muscles was measured, but the total area of the longitudinal muscles was computed from measurements of both width and height. A record of the total number of sections containing vertical wing muscles was made. The final quantitative rating of the wing muscle development was based largely on the width of the vertical muscles, for the correlation between width of muscle and number of sections was close enough to make the latter measurement of value largely as a check on the former. A careful comparison of the longitudinal and vertical muscles revealed that the longitudinal, proportionately, were consistently less developed than the vertical in intermediate forms. Because the correlation between the two sets of muscles proved to be rather constant, little was to be gained by using the longitudinal muscles as a quantitative measure of intermediacy. That there might be a close correlation between these two groups of muscles seemed probable from a study of their development in the early embryogeny of the aphid, for it was learned that both appear at about the same time. At least, when the vertical muscles were found the longitudinal were always present, and both showed about the same relative development. This made it doubtful whether there was a difference in the time of determination for these two different sets of muscles, although there could be.

The differential characters which proved of greatest worth as measures of intermediacy in this investigation were the wings, ocelli, and wing muscles, and consequently these were most carefully rated. After the raw data on these structures had been obtained they were compared with the mean for the same organs in what were considered typical winged individuals. In tables of these values the development of these differentiating structures is expressed in percentages.

By means of the differentiating characters described above, a diagnosis of the intermediates of this study is made with the results presented in Tables I to IX. The data are grouped according to conclusions to be drawn, but for the present this may be ignored. The reasons for so arranging the data that conclusions are anticipated is that it saves repeating this material in a general table.

Expected Composition of Intermediates Under Various Suppositions as to Order of Determination and Direction of Change

As seen above, Goldschmidt has applied the time-of-determination theory to intersexes. In accordance with that theory, organs which are the first to be changed in successive offspring must be the latest to be determined in development, while the latest to change in successive offspring are the earliest to be determined. The order of embryonic segregation is the reverse of the order of modification in successive offspring. If this theory is applicable to intermediate aphids produced during a transition from winged to wingless or wingless to winged, then it should be possible to determine the order of segregation if the direction of change is known.

Whether the time-of-determination theory is correct in a given instance cannot be proven by finding that all characters of the two types of individuals change simultaneously. In fact, such a circumstance would make possible the supposition that there was no difference in the time of determination of the different organs. But even though several structures did appear at the same time in the embryo, the possibility remains that they may not all have been determined at the same moment, as there could be a difference in the intervals between the time of physiological determination of two or more organs and their morphological appearance, and that difference might make possible their simultaneous appearance in development even though their determinations coincided in time. Nor is the time-of-determination theory confirmed by the discovery that certain characters change in earlier offspring than do others. If intermediates that are near to the one extreme type always possess a given set of characters, and those near the other extreme type always have another set of characters, these facts furnish no support to the time-of-determination hypothesis. What is needed to prove the theory is to demonstrate (1) that when the intermediate condition is approached from one of the extremes, the changes in successive offspring shall take place in a definite order, and (2) that when the intermediate condition is approached from the opposite extreme the same order of change shall occur.

Whether the conditions necessary for confirmation of the time-of-determination theory are met in a given case of intermediacy can be tested by (1) considering all the possible types of individuals that would result from all conceivable orders of determination of the several differentiating structures concerned in intermediacy, and (2) tabulating the data on the quantitative development of each differentiating structure in all the intermediates obtained. Then test which assumption each intermediate best fits. If more intermediates fit one assumption than others, that assumption is favored. With three different organs (wings, ocelli, and wing muscles) to be considered there are six different possible orders of embryonic determination which are shown below in the diagram as numbered assumptions. The order of determination is indicated by the arrows, that is, in I the wings are determined first, then wing muscle, and the ocelli last.

Six different possible orders of determination for three differentiating organs

Assumption I.	Wings	⟶	Wing Muscle	⟶	Ocelli
Assumption II.	Ocelli	⟶	Wing Muscle	⟶	Wings
Assumption III.	Wing Muscle	⟶	Ocelli	⟶	Wings
Assumption IV.	Wings	⟶	Ocelli	⟶	Wing Muscle
Assumption V.	Ocelli	⟶	Wings	⟶	Wing Muscle
Assumption VI.	Wing Muscle	⟶	Wings	⟶	Ocelli

For a given order of determination there will be a number of intergrades as illustrated below in Assumptions IV A and IV B. The " A " part of Assumption IV shows the various kinds of intermediate-winged aphids which would be produced as a result of a change from winged to wingless forms, with the order of determination wings, ocelli and wing muscle. Assumption IV B indicates the different kinds of intermediate-winged aphids produced with the same order of determination as in Assumption IV A, but during a transition from wingless to winged types. The third intermediate from the top of Assumption IV A would deviate from a normal winged individual by possessing normal wings, slightly reduced ocelli and much reduced wing muscle. If a detailed chart of each of the six assumptions is made out and then an attempt is made to determine which of the six possibilities for embryonic determination each intermediate best fits, it is obvious that each intermediate form will fit equally well two of the possibilities, depending on the order of determination, as will be illustrated later. If more intermediates fit one assumption than others, that assumption is favored. The above procedure was followed with the intermediate-winged aphids of this investigation. Three structures, wings, ocelli and wing muscles, showed enough intergradations in development to make them satisfactory for such a study. As previously stated, an effort was made to discover to which one of the six different possible orders of embryonic determination the intermediate-winged aphids of this research tabulated in Tables I to IX best conformed.

It will be seen that for each possible order of embryonic segregation there are two ways in which the characters may be combined, depending on the direction of change. The diagrams below illustrate the different combinations of intermediate characters as they occur in different winged-wingless aphids for selected assumptions. Each diagram shows a complete transition from typical winged to wingless forms or vice versa. Read from left to right to determine the nature of any given intermediate.

If the transformation is from winged to wingless with the order of determination wings, ocelli and wing muscle, Assumption IV A, the wings would be most developed, the ocelli less, and the wing muscles least. If, however, the transition is from wingless to winged with the order of determination the same as in Assumption IV, then the wings will be least developed, the ocelli more, and the wing muscle most as shown in Assumption IV B. Thus it is possible to have two graduated series, one showing intermediates resulting from a change from winged

Assumption IIIA Regarding Time of Determination

Direction of change—winged to wingless

Combinations of intermediate characters				
Wing Muscle	→	*Ocelli*	→	*Wings*
Normal		Normal		Normal
Normal		Normal		Slightly reduced
Normal		Slightly reduced		Much reduced
Slightly reduced		Much reduced		Very much reduced
Much reduced		Very much reduced		None
Very much reduced		None		None
None		None		None

Assumption IIIB Regarding Time of Determination

Direction of change—wingless to winged

Combinations of intermediate characters				
Wing Muscle	→	*Ocelli*	→	*Wings*
None		None		None
None		None		Slightly developed
None		Slightly developed		More developed
Slightly developed		More developed		Highly developed
More developed		Highly developed		Normal
Highly developed		Normal		Normal
Normal		Normal		Normal

Assumption IVA Regarding Time of Determination

Direction of change—winged to wingless

Combinations of intermediate characters				
Wings	→	*Ocelli*	→	*Wing Muscle*
Normal		Normal		Normal
Normal		Normal		Slightly reduced
Normal		Slightly reduced		Much reduced
Slightly reduced		Much reduced		Very much reduced
Much reduced		Very much reduced		None
Very much reduced		None		None
None		None		None

ASSUMPTION IVB REGARDING TIME OF DETERMINATION

Direction of change—wingless to winged

Combinations of intermediate characters				
Wings	⟶	*Ocelli*	⟶	*Wing Muscle*
None		None		None
None		None		Slightly developed
None		Slightly developed		More developed
Slightly developed		More developed		Highly developed
More developed		Highly developed		Normal
Highly developed		Normal		Normal
Normal		Normal		Normal

to wingless, and another including totally different kinds of intermediates when the change is from wingless to winged. The aphids of these two series, Assumptions IVA and IVB, are complementary in their composition; that is, in one the wings are large, in the other small, and the same relationship holds for the other structures. Figures 7 and 8 picture this complementary condition in the wing muscles; Fig. 7 is an intermediate with wings .60, and degenerate wing muscle .03 developed, while Fig. 8 is rated with wings .22 and non-degenerate wing muscle .66.

As indicated above, any intermediate aphid should fit equally well two of the six assumptions for different orders of determination because an intermediate of a structural composition that should result from an assumed order of determination, such as wing muscle, ocelli, wings, Assumption III B, could also result from the reverse order of determination, wings, ocelli, wing muscle, Assumption IV A, if the direction of change were reversed. The above-mentioned assumptions illustrate this, for the composition of an aphid which fits Assumption III B is identical with one which fits Assumption IV A. In other words, an intermediate produced as a result of a transition from winged to wingless in Assumption IV A is the same in composition as one resulting from a conversion from wingless to winged in Assumption III B. Hence, all that is shown by such a classification and testing of data would be that the order of determination was one of two possibilities, one the reverse of the other, and unless the direction of change is known the elimination of one of these possibilities is out of the question.

It was not known from the conditions under which these intermediate-winged aphids were reared whether a given intermediate was produced as a result of change from winged to wingless or the reverse. Unlike the gamic-parthenogenetic intermediates found by Shull, there was no gradual change from one type to the other. Shull found it possible to get stocks producing mostly gamic females and then effect a gradual change to parthenogenetic types with many intermediates appearing during the change. Presumably the intermediates produced under these conditions were the result of a gamic-to-parthenogenetic change. In the experiments in which intermediate-winged forms appeared there were usually both winged and wingless aphids with a few intermediates. Several females were placed on one plant and one aphid might be giving birth to offspring which were intermediate as the result of change in one direction while another could be producing intermediates as a result of change in the opposite direction. And even if a female were to be reared in isolation on a plant, little could probably be told about the direction of change which resulted in intermediacy for the winged and wingless offspring occur in a very erratic manner through the family. It is also conceivable that in a given experiment an aphid could produce intermediate offspring as the result of a change in one direction at one time, and other intermediates from the opposite direction of change at another time. If this occurred there would be no way by which the direction of change could be detected from the daughters. Shull (1928, 1929) has shown that light and temperature modify the development of wings but these agents cannot be relied on to change the course of development in all individuals in the strains now available which are producing the intermediates. Thus it will be seen that the experiments themselves afford no reliable method whereby the direction of change can be ascertained.

However, this investigation has revealed what is considered to be a means of deciding the direction of change which results in intermediacy. As has been stated above, there were found to be two different classes of intermediates based on internal structures, namely, (1) those with histologically normal wing muscles and (2) those with degenerative wing muscles. It is postulated by the author that degenerate muscles are the consequence of a transformation from winged to wingless forms. Development is assumed to have begun with factors favoring wing production dominant, then a physiological change taking place which not only inhibited further development of characters associated with wings, but brought about more or less degeneration of the wing muscle already produced. When a change takes place in the opposite direction, that is, a conversion from wingless to winged, there is no degeneration of the organs of the winged type because in this transition the physiology changes from one in which those organs are not present to one which favors their development. With conditions favorable for the production of the structures characteristic of winged females, these organs undergo normal histological development. The facts are that about 56 per cent of the intermediates of this investigation were characterized by normal muscles, while about 44 per cent possessed degenerate muscles. This interpretation of the two classes of intermediates based on the nature of wing muscle affords a means of determining the direction of change in a given individual so that it is possible to classify each as the result of a conversion from winged to wingless or wingless to winged. In Tables I to IX inclusive, all aphids listed under the " A " assumptions possess degenerate wing muscles; all those listed under the " B " assumptions are wingless-to-winged transformations.

By a study of these Tables (I to IX inclusive) the conclusion that normal and degenerate wing muscles are correlated with some direction of change is deduced from the way in which the tabulated material fits the various assumptions. If degeneration of muscles were a purely fortuitous phenomenon, with one aphid as likely as another to be affected regardless of the direction of transformation producing intermediacy, every Assumption " A," of which there are six, should include some intermediates, which according to the conditions of their classification would possess degenerate muscles. This expectation is not realized. Moreover, Assumptions III and IV are complementary as discussed above, and about 50 per cent of the aphids of this investigation conform to Assumption IV B; yet there is not an aphid to be found conforming to its complementary Assumption III A. If degeneration of wing muscle were determined by mere chance, it would seem very probable that some of the aphids classified in Assumption IV B would have come under the influence causing muscle degeneration, thus changing their classification to Assumption III A. The converse of this statement is equally true; if the wing muscle in intermediates postulated to be changing from wingless to winged were decided only by chance, there should also be aphids which would fit Assumption III B; but none were found. There seems no other way to explain why aphids with the intermediate characters of Assumption IV B, normal muscle, should not have possessed degenerate muscles which would fit them to Assumption III A except that normal muscle is associated with a certain direction of change which is here postulated to be from wingless to winged. Thus it will be seen that the histological character of intermediate-winged aphids is correlated with direction of change. Whether it means a winged-to-wingless or wingless-to-winged transition depends on the correctness of the theory that degenerating muscles result from a change in physiology to which the immature wing muscles are susceptible.

This method of establishing the direction of change which results in intermediacy makes it possible to eliminate one or the other of Assumptions III and IV which are complementary as far as the development

TABLES I–IX

Tables I, II, III, IV, V, VI, VII, VIII and IX contain the data regarding the development of the differentiating organs of intermediacy of winged-wingless aphids. The data are arranged, in so far as possible, to fit the various assumptions made.

TABLE I

Composition of intermediate aphid fitting Assumption I A. Order of determination: wings→wing muscle→ocelli. Change from winged to wingless. Degenerate wing muscle.

Slide No.	Characters to which Assumption I A is fitted			Number of sensoria	Antennal color
	Wing length	Wing muscle development	Ocellar development		
1196 c (7)	.50	.49	.41	5–6	0

TABLE II

Composition of intermediate aphid fitting Assumption I B. Order of determination: wings→wing muscle→ocelli. Change from wingless to winged. Normal wing muscle.

Slide No.	Characters to which Assumption I B is fitted			Number of sensoria	Antennal color
	Wing length	Wing muscle development	Ocellar development		
1170 c (2)......	.03	.28	.35	5–4	0

TABLE III

Composition of intermediate aphids fitting Assumption II A. Order of determination: ocelli→wing muscle→wings. Change from winged to wingless. Degenerate wing muscle.

Slide No.	Characters to which Assumption II A is fitted			Number of sensoria	Antennal color
	Ocellar development	Wing muscle development	Wing length		
1083 f	.35	.23	.15	4–8	0
226 e	.35	.16	.10	6–7	0
245 f	.23	.20	.10	6–8	0
288 e (4)	.15	.12	.08	5–5	0
288 e (5)	.11	.06	.05	5–6	0
909 a	.23	.08	.04	6–5	0
40 c (2)	.22	.20	.02	7–7	0
905 b (4)	.35	.16	.02	10–5	1
905 b (5)	.35	.33	.02	10–5	1
1016 a	.35	.20	.01	4–4	0
1087 a (4)	.17	.16	.01	5–5	0

TABLE IV

Composition of intermediate aphid fitting Assumption II B. Order of determination: ocelli→wing muscle→wings. Change from wingless to winged. Normal wing muscle.

Slide No.	Characters to which Assumption II B is fitted			Number of sensoria	Antennal color
	Ocellar development	Wing muscle development	Wing length		
490 g (1)	.66	.91	.95		3

TABLE V

Composition of intermediate aphids fitting Assumption IV A. Order of determination: wings→ocelli→wing muscle. Change from winged to wingless. Degenerate wing muscle.

Slide No.	Characters to which Assumption IV A is fitted			Number of sensoria	Antennal color
	Wing length	Ocellar development	Wing muscle development		
P–27	1.00	.95	.90		
285 e (2)	.80	.59	.12	5–6	0
285 e (1)	.80	No sections of head	.23	6–6	0
286 f	.80	.35	.08	7–7	1
282 e	.75	.50	.49	8–8	2
288 e (1)	.75	.47	.12	5–6	0
1196 c (2)	.70	.47	.28	5–7	0
428 d (2)	.70	.59	.45		
235 b	.65	.59	.57	13–13	3
296 b (3)	.60	.46	.08	7–7	0
285 c (2)	.60	.46	.28	7–8	1
279 e	.60	.46	.06	7–7	1
220 e	.60	.46	.03	6–5	0
1085 g (1)	.60	.46	.41	6–?	1
1196 c (11)	.60	.41	.23	6–6	0
488 d (2)	.50	.46	.41		0
220 d	.50	.35	.01 –	6–6	0
1021 a	.50	.46	.25	5–4	0
1188 b	.50	.46	.16	9–6	0
282 c (1)	.40	.33	.08	7–7	1
212 b	.40	.33	.08	5–6	0
1130 f	.40	.33	.16	5–6	0
280 c (2)	.35	.23	.01 –	7–8	2

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TABLE VI

Composition of intermediate aphids fitting Assumption IV B. Order of determination: wings→ocelli→wing muscle. Change from wingless to winged. Normal wing muscle.

Slide No.	Characters to which Assumption IV B is fitted			Number of sensoria	Antennal color
	Wing length	Ocellar development	Wing muscle development		
1168 a	.60	.71	.83	5–8	0
472 c (12)	.60	.71	.74		0
1061 e (1)	.50	.94	.99	14–15	4
307 a (10)	.50	.59	.74	11–11	3
472 c (3)	.40	.46	.90		0
1196 c (4)	.40	.46	.66	9–8	1
597 d (6)	.35	.46	.83	6–6	0
1196 c (1)	.30	.46	.58	6–6	0
44 c (2)	.25	.46	.49	7–7	1
1077 a	.25	.71	.74	5–6	0
905 b (3)	.25	.35	.45	5–8	0
1063 g (2)	.22	.46	.66		1
414 c	.20	.35	.63	6–6	0
472 c (1)	.20	.46	.57		0
1087 g (1)	.20	.46	.62	5–6	0
1174 c	.12	.35	.41	5–5	0
1097 f	.10	.71	.99	12–9	3
717 b	.10	.59	.70	7–7	3
1015 f	.10	.59	1.00	11–7	0
472 c (4)	.09	.35	.66		0
44 c (3)	.08	.35	.46	7–8	1
472 c (8)	.05	.35	.49		0
895 d	.04	.23	.37	6–8	0
267 d	.04	.11	.23	6–6	0
597 d (1)	.04	.11	.33	6–6	0
472 c (14)	.03	.35	.42		0
589 d (3)	.03	.23	.42	6–7	1
44 c (1)	.03	.23	.42	4–4	1
44 c (4)	.02	.35	.40	8–8	1
589 d (4)	.02	.33	.62	7–5	0
597 d (2)	.02	.23	.33	6 or 7	0
569 d (3)	.02	.23	.25		
295 c (2)	.02	.11	.25	7–8	0
905 b (1)	.02	.35	.49	6–7	1
569 e (1)	.015	.23	.25		
1111 a (1)	.01	.23	.33		1
1063 g (1)	.01	.23	.41	5–5	0
1035 d (9)	.01	.20	.33	6–7	0
1079 g (3)	.01	.23	.37	5–5	0
1079 g (4)	.01	.23	.37	6–6	0
1079 g (5)	.01	.28	.41	5–5	0
1079 g (6)	.01	.17	.37	5–7	0
1079 g (7)	.01	.23	.28	4–7	0
1035 d (1)	.01	.23	.28	5–7	0

TABLE VI (*continued*)

Composition of intermediate aphids fitting Assumption IV B

Slide No.	Characters to which Assumption IV B is fitted			Number of sensoria	Antennal color
	Wing length	Ocellar development	Wing muscle development		
589 d (8)	.01	.28	.37	7–6	0
589 d (7)	.01	.23	.29	7–7	0
589 d (5)	.01	.17	.29	8–7	1
1103 d (1)	.01	.23	.37	6–6	0
1103 d (3)	.01	.23	.37	5–5	0
1103 d (4)	.01	.23	.33	5–5	0
1103 d (5)	.01	.35	.49	4–4	0
1087 g (2)	.01	.23	.41	5–4	0
1085 d (8)	.01 –	.11	.37		0
1035 d (6)	.01 –	.23	.33	3–5	0
1035 d (7)	.01 –	.23	.33	4–6	0
1035 d (5)	.01 –	.11	.23	8–5	0
307 a (7)	0	.46	.58	13	4
773 b	0	.59	.74	8–9	2
308 a (4)	0	.70	.72	12–13	3
454 b	0	.82	.91		
307 a (3)	0	.58	.66	13	4

TABLE VII

Composition of intermediate aphids fitting Assumption V A. Order of determination: ocelli→wings→wing muscle. Change from winged to wingless. Degenerate wing muscle.

Slide No.	Characters to which Assumption V A is fitted			Number of sensoria	Antennal color
	Ocellar development	Wing length	Wing muscle development		
1017 c	.94	.45	.33	5–9	
224 c	.35	.30	.01 –	6–7	0
227 d	.35	.20	.01 –	6–7	1
1162 c	.59	.20	.01 –	6–6	0
44 c (6)	.23	.15	.01 –	5–7	1
287 e (1)	.35	.15	.01 –		
44 c (5)	.23	.10	.08	5–7	0
219 c	.46	.10	.01 –	5–5	0
228 e	.35	.10	.01 –		
295 d (1)	.23	.06	.01 –	6–6	0
296 b (2)	.23	.05	.03	6–6	0
40 c (3)	.23	.05	.04	6–6	0
270 e (1)	.11	.03	.01 –	6–7	0
295 c (3)	.11	.02	.01 –	6–7	2
887 d	.35	.02	.01 –	6–5	0

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TABLE VIII

Composition of intermediate aphids fitting Assumption V B. Order of determination: ocelli→wings→wing muscle. Change from wingless to winged. Normal wing muscle.

Slide No.	Characters to which Assumption V B is fitted			Number of sensoria	Antennal color
	Ocellar development	Wing length	Wing muscle development		
1091 d (1)	.47	.70	.83	7–7	1
1091 d (2)	.47	.70	.86	9–9	1
589 d (2)	.59	.60	.67	9–10	1
1089 g	.33	.45	.58	7–11	0
597 d (5)	.33	.40	.49	5–4	0

TABLE IX

Composition of intermediate aphids not fitting any time-of-determination assumption. Degenerate wing muscle.

Slide No.	Characters to which no Assumption is fitted			Number of sensoria	Antennal color
	Wing length	Ocellar development	Wing muscle development		
214 e	.40	.40	.33	5–6	0
490 g (3)	.01 –	.23	.01 –		
P-39	.01 –	.11	.01 –		
490 g (2)	.01 –	.23	.01 –		0

of the several organs is concerned. For example, aphids conforming to Assumption III B show the same composition as those conforming to Assumption IV A; they differ only in the direction of change as established by the histological character of the wing muscle. In Assumption III B, the change is from wingless to winged; in Assumption IV A it is from winged to wingless. If degenerate muscles are considered the result of a change from winged to wingless, an individual with a given composition fitting equally well both Assumptions III B and IV A would be placed in IV A if the muscles were degenerate. There is one outstanding piece of evidence that degenerate wing muscles do indicate a transition from winged to wingless aphids. Comparing the first few aphids tabulated in Tables V and VI, it will be observed that the wing development is what one would expect if the wings were determined first as here proposed. At the top of Table V (winged to wingless) is listed an intermediate with fully developed wings, which is possible if wings are determined before wing muscles, but could not occur if muscles were determined before wings. On the other hand, the first aphid listed in Table VI (wingless to winged) shows only .6 of normal wing length. It is probably not without significance that the first few aphids listed in Table V show considerably more wing development than the first few listed in Table VI. If wings are determined first, then in a winged-to-wingless transition the wings might be expected to show on the whole more development than in a wingless-to-winged change. The other possibility would be to postulate that wings are determined last, which does not fit the facts, as such an hypothesis would rule out the combination of fully developed wings and reduced wing muscle. More than that it is hardly conceivable even in an aphid with the composition shown by No. 285 e (2), Table V, wing length .8, ocellar development .59, and wing muscle development .12, that wing muscle could have been determined first, which the alternative theory would require. Thus all available evidence points to the conclusion that wings are determined before wing muscle, and that degenerate wing muscle characterizes a transformation from a winged to a wingless aphid in development.

So far in this paper no attempt has been made to prove that the ocelli are segregated at a time between the wings and wing muscles. Some evidence for this position of the ocelli in the order of determination is available. To arrive at this evidence it will be helpful to show what would occur in the event that ocelli were determined between wing and wing muscles at an arbitrarily set time. In the chart below it is assumed that the order of determination is wings, ocelli, and wing muscle. There is also the assumption made that no overlapping in times of segregation for the several organs occurs. The times indicated are purely hypothetical as nothing is known about the actual time involved.

A.

Change from winged to wingless

Wing determination	Ocellar determination	Wing muscle determination
1:00 o'clock	2:30 o'clock. Change to wingless	3:00 o'clock
Fully developed	One-half developed	No development

B.

Change from wingless to winged

Wing determination	Ocellar determination	Wing muscle determination
1:00 o'clock	2:30 o'clock. Change to winged	3:00 o'clock
No development	One-half developed	Fully developed

The ocelli in *A* are only one-half developed because a change in physiology unfavorable to their development took place when they were only half segregated in the embryogeny. In *B* the ocelli are only one-half developed because a change in physiology which made their development possible in the embryogeny did not occur until the period of determination was half completed.

From this illustration it is evident that under the conditions postulated, the ocelli may not be very different in amount of development in two aphids derived from opposite directions of change, but the wings and wing muscles would show a considerable difference. If again Tables V and VI are compared it will be found that the facts conform to the theory. For example, Table V, slide 285 e (2), wing length .80, ocellar development .59, wing muscle development .12, compared with Table VI, slide 307 a (10), wing length .50, ocellar development .59, wing muscle development .74, shows exactly the same relationship as illustrated above. Not all of the aphids fit the conditions as perfectly as the ones drawn for illustration but the general trend is clearly the same.

Thus the facts support the theory that of the three organs, wings, ocelli, and wing muscle, the ocelli are median in the time of embryonic segregation.

If the time-of-determination theory were perfectly applicable to intermediate-winged aphids, and the intermediates were to be classified according to the assumptions of Tables I to IX inclusive, which are based on the time-of-determination theory and the direction of change established by the character of the muscle, then all intermediates would meet the conditions which parts "*A*" and "*B*" of one assumption entail. Examination of Tables I to IX, which are an attempt to discover the applicability of the time-of-determination theory when the foregoing conditions are imposed, shows that the material does not all fit any one assumption. However, Assumption IV is applicable to about 69 per cent of the aphids of this research. Other assumptions which are applicable to intermediates are I (2 per cent), II (10 per cent), and V (16 per cent). This would suggest that the time-of-determination theory has at least a limited applicability to the development of intermediate-winged aphids. In fact, the large percentage conforming to this scheme probably means that the assumption that the composition of intermediates is determined by the times of embryonic segregation of their differential structures is in a general way correct. Were there no such relation between the organs of intermediacy a much smaller proportion of intermediates should be of the composition shown in Tables V and VI. As previously stated, there are 6 conceivable assumptions for these intermediates. If the combinations were entered into at random only one-sixth of them should be of the kinds listed in Assumption IV (Tables V and VI). Their actual numbers are over four times this expectation.

Besides the 84 intermediates which conform to a single assumption regarding times of determination (Tables V and VI), there are 34 which fit other assumptions (Tables I, II, III, IV, VII, and VIII), and 4 that could not be classified under any one of these schemes (Table IX). It will be noted concerning these aphids not meeting the conditions of Assumption IV that most of them have been placed in another assumption due to the fact that they appear to show a shift in the time of determination of the ocelli. In most instances the development indicates a determination of ocelli before wings, while in two cases the amount of ocellar development indicates their determination after the wing muscles. This variation of the indicated time of determination of the ocelli in both directions suggests again that the usual time of segregation is between the wings and wing muscles.

The simplest explanation of the irregularities in the order of determination found in Tables I, II, III, IV, VII, and VIII, after eliminating certain possible errors, is to postulate that there actually is variation in the time at which segregation takes place. The variation on at least a few occasions is so great that the time when the wings are determined is changed from the first of the three to the last. However, of particular interest is the fact that in all of the material studied not one case was found in which wing muscles were indicated to be determined first. It may be significant that there are far more individuals in which there was a change in the usual time of determination of only two structures as contrasted with those in which the position of all three was disturbed. For it would seem reasonable if this explanation is correct that slight irregularities would occur more frequently than those which involved more radical departures from the usual. The small group of four aphids (Table IX), in which two organs are of about the same development are exceptional individuals in which a change in the time of segregation of only one structure may have caused the irregularity. That the time of determination is not an unalterable fixed thing is shown not only by these irregular classifications, but in Tables V and VI, to which the intermediate group as a whole best conforms, there is considerable variation among the individuals listed.

An Interpretation of the Combinations of Characters in Intermediate Aphids Based on the Physiological Level Theory

Inasmuch as the combinations of characters in the intermediate aphids of this report do not conform perfectly to the time-of-determination theory, an analysis based on the physiological level theory would seem indicated. It will be remembered from an earlier statement that this theory postulates that the degree of intermediacy depends upon the state of some physiological process during development. It could depend on the quantity of some substance (possibly a hormone) present during the embryogeny. If all the differentiating structures reacted to exactly the same extent to a given quantity of this substance, or to the " level " of this physiological process, there would not appear to be a linear order of determination; all structures would give evidence of having been determined at the same time, or during the same part of the embryogeny. According to this theory without modification, when a given degree of intermediacy in one structure occurs, each other organ should show a definite degree of intermediacy. For example, if one intermediate at a given physiological level shows .1 wing, .2 ocellar, and .4 wing muscle development, every intermediate with .1 wing development should have the same grade of ocellar and wing muscle development. If, however, wings start development at a given physiological level, let us say when there is a certain concentration of a substance, while ocelli and wing muscles do not, there arises a situation in which a difference in the time of determination of these organs is surmised, although such a difference may not exist. In other words, there may be a difference in the threshold of stimulation of the intermediate structures causing their appearance in different degrees. This explanation denies any importance to differences in the time of embryonic segregation of differentiating parts. There could be such differences even if there were a difference in the threshold of stimulation of the several organs, but it would not be the differences in time of determination of the different parts that would determine the composition of intermediate individuals, rather it would be the differences in the threshold of stimulation of the differentiating parts.

A casual examination of the tables of intermediate-winged aphid data indicates at once that the physiological level theory without modifications is not applicable to these aphids, for there is apparently no constant relationship between the degrees of development of the several structures such as this theory requires. But if it is assumed that there is a difference in the threshold of stimulation of the several organs in different individuals then there should be a difference in the development of a given structure in different intermediates. If Tables V and VI, which include nearly 70 per cent of the material of this investigation, are studied, it will be found, to take a typical example from Table V, slide 1196 c (11), that wings are .6, ocelli .41, wing muscle .23 in development. This would mean, according to the physiological level hypothesis, that the wings possessed a lower threshold of stimulation than either of the other two structures. Now if a comparison is made with Table VI, slide 1168 a, in which the wings are .6, ocelli .71, and wing muscle .83 developed, it will be seen that in this case the wing muscle would have to possess the lowest threshold of stimulation, ocelli the next lowest, and the wings the highest. It will be noted in the latter illustration there is required a reversal of the order of relative levels in the thresholds of stimulation of the several structures; the threshold of stimulation of the wings in the second illustration is highest, whereas in the first illustration it was lowest. Thus, if this proposal of a varying threshold of stimulation is offered as a modification of the physiological level hypothesis in explanation of the combination of characters in the majority of intermediates in this work, it is necessary to postulate not only that the threshold of stimulation is different in different organs for the same individual, but that it is different for the same organ in different individuals, and that for most intermediates there is an alternating though definite order in which the threshold of stimulation varies. Other aphids which do not conform to Tables V and VI would necessitate the assumption of an order in which the thresholds of stimulation in the several organs vary. It is conceivable that the thresholds of stimulation could vary for the several structures in different aphids in such a manner as to produce a series fitting the time-of-determination scheme, but it seems highly improbable that at one time the threshold would so vary as to give individuals of a composition that would conform to the winged-to-wingless direction of change and at another time individuals of such a composition that they would fit a wingless-to-winged direction of change. The difficulties involved in making this assumption provide the greatest objection to this hypothesis.

Discussion

With the exception of the work of Shull on aphids, most of the previous investigations concerning intermediacy in insects have dealt with intersexes, that is, forms intermediate between the male and female of the species. The chief contributor in this field has been Goldschmidt (1931), who, in an analysis of intersexuality of the gipsy moth, *Ly-*

mantria dispar, considers intersexuality in these moths as depending chiefly on general sex genes.

The intermediacy of aphids which has been a subject of considerable study at the University of Michigan is in sharp contrast in some respects to that of the moth reported by Goldschmidt. In the first place, the type of intermediacy which we have studied in aphids is not an intersexual phenomena, for we are dealing with only one sex. Secondly, it is highly improbable that it is due to a quantitative genic influence as postulated for *Lymantria,* for the aphids of this research were all parthenogenetic females, and in a parthenogenetic line there is a minimum of opportunity for genetical variation. Certainly the differences in individuals of a single strain of a parthenogenetic species are less likely to result from genetical causes than in an animal which reproduces bisexually as in the gipsy moth where different geographical races were involved in the crosses. Inasmuch as it has been found possible by modification of the environment to change the percentage of winged to wingless aphids, it would seem probable that intermediacy was due to some physiological state induced by environmental factors. However, Goldschmidt's assumption that embryonic determinations occur at different times in development does with modifications seem applicable to aphids, as has been discussed above. Modifications from a perfect application of the time-of-determination theory to aphids are necessary because the data from intermediate-winged aphids not only show considerable variation in the times of determination within the usual order of embryonic segregation but this sometimes is disturbed by irregularities.

Thus it will be seen that in two types of intermediacy which are quite different in that one is of an intersexual character and results from a genic imbalance, the other not intersexual and probably due to environmental factors, yet both support in a general way the theory that in at least some of the invertebrates there is a succession of embryonic determinations.

SUMMARY

In two strains of aphids of the species *Macrosiphum solanifolii,* intermediates between winged and wingless females were experimentally produced, and a histological study was made of these. Aphids may show internal intermediacy when there is no evidence of it externally, therefore the degree of winged-wingless intermediacy cannot be safely judged by external observations alone.

Two theories attempting to explain the combination of characters (wings, ocelli, wing muscle) found in intermediate-winged aphids are discussed. The first theory postulates that the combination of characters in intermediates is due to the fact that the differentiating organs are determined at different times in the embryogeny (time-of-determination theory), while the second theory assumes that the structural character of intermediates results from a given concentration of some substance (physiological level theory) in development.

It could not be ascertained from the conditions under which these aphids were reared what direction of change (winged to wingless or wingless to winged), assuming there was a change, produced a given winged-wingless intermediate, but evidence is given which indicates that the character of the wing muscle is correlated with some definite change in the direction of embryonic development. The evidence makes it appear probable that degenerate wing muscle means that the intermediate aphid is the result of some physiological change from a winged to wingless form during the embryogeny; whereas the intermediate with normal wing muscle appears to be the result of a change from wingless to winged during its development.

If the time-of-determination theory were perfectly applicable to intermediate-winged aphids they should all fit one (the same one) of the six possible assumptions for the time at which the wings, ocelli and wing muscles may be determined in the embryogeny. More than two-thirds of the aphids of this study did fit one assumption (Assumption IV) which is evidence that in a general way the composition of intermediates is determined by the segregation of their differentiating structures at different times in the embryogeny. This indicates that organs are determined in a serial order in aphids, as has been found true for certain vertebrates. The order of embryonic determination of the structures distinguishing winged from wingless is wings, ocelli, and wing muscles.

The small differences between the amount of development in wings, ocelli, and wing muscles found in many cases is taken to indicate that the time of determination of these organs is close together. However, that the period of embryonic segregation for these three structures is of considerable duration is suggested by the large number of intergrades.

Most of the intermediates which did not conform to Assumption IV appeared to owe their irregular combination of characters to a shift in the time of determination of the ocelli. The simplest explanation of these irregularities is to postulate that the time of segregation may vary for a given organ in different individuals.

An interpretation of the combination of differentiating characters of intermediate-winged aphids based on the physiological level theory would involve the necessity of assuming that the threshold of stimulation for the development of these organs varies in different individuals. Moreover, the threshold of stimulation would have to show a series of regular variations to produce aphids fitting one direction of development, as winged to wingless, at one time and another direction, as wingless to winged, at another time. The difficulties involved in making such an assumption renders this hypothesis highly improbable.

The results of this investigation support the time-of-determination theory with the modification of frequent variations in the time at which an organ may be embryonically determined.

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PHYSIOLOGY OF REPRODUCTION OF OSTREA VIRGINICA

I. Spawning Reactions of the Female and Male

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Sexual cycles of many marine organisms are determined by specific rhythms in the development of their gonads, and by environmental conditions which to a great extent control the discharge of ripe sex cells. The simplest sexual reactions are to be found among the animals which, lacking organs of copulation, shed their eggs and sperm directly into the water. In these cases fertilization is a matter of the chance meeting of ovum and spermatozoan outside of the organism, and the propagation of the species is dependent upon the production of large numbers of sex cells and the correct timing of their emission. The latter condition is significant for it enhances the probability that the eggs will be inseminated before they become unfertilizable. Since our knowledge of the physiology of the reproduction of marine invertebrates is very inadequate, conditions which govern the discharge of their sex products are but little understood.

The present investigation of the physiological reactions involved in the reproduction of the oyster was undertaken with the view of determining the factors controlling the sexual activity of this mollusk. Because of its widespread distribution, its abundance and the anatomical simplicity of its organs of reproduction, the common oyster of commerce appears to be very suitable for such a study. The work was carried on during a number of summers at Woods Hole, Massachusetts; Milford, Connecticut; and Galveston, Texas. Supplementary observations on *O. gigas* (Japanese oyster) and *O. commercialis* (Australian oyster) were made at the Hopkins Marine Station, Pacific Grove, California, and at the Marine Laboratory of the University of Hawaii in Honolulu. The first part of this study contains a description of the sexual reactions of the female and the male oyster. The subsequent parts, which will be published separately, deal with the stimulation and specificity of sexual reactions and their significance for the survival of the species.

Reproductive Cycle

The annual reproductive cycle in *Ostrea virginica* begins in autumn immediately after the completion of the preceding spawning period. At this stage the gonad follicles are lined with indifferent germinal epithelium and the sexes are often indistinguishable. A transition to the next, the sex-differentiation stage, is marked by a slight expansion of the gonad tissue and the appearance of either ovo- or spermocytes. The gonad remains in this condition until the next spring when an extensive proliferation of the follicles accompanied by rapid ovo- or spermatogenesis results in the production of large numbers of sex cells. During the following summer almost all of them are discharged and the few remaining within the follicles are cytolized and destroyed by phagocytes.

The spawning season, during which one or several emissions may occur, lasts for about six weeks in the North Atlantic States (north of the Chesapeake Bay) but in the warm waters of the South Atlantic and Gulf of Mexico may extend from March to the end of October. In every body of water a great part of the oyster population spawns more or less simultaneously. This is evidenced by the appearance in plankton collections of a large number of oyster larvæ which often occur at regular intervals or cycles corresponding to the outbursts of sexual activity (Prytherch, 1928; Hopkins, 1931).

Young oysters, excepting those living north of Cape Cod, reach sexual maturity at the end of their first year (Coe, 1936) and barring unfavorable conditions, the mollusk breeds every year until its death. The fecundity of an oyster apparently does not decrease with age for in very large specimens measuring from 6 to 10 inches in length, the gonads may be well developed and contain large numbers of fertilizable eggs.

Anatomy of the Organs of Reproduction

A brief description of the essential anatomical features of the gonads is necessary for an understanding of the mechanism of ovulation and ejaculation. In both sexes the gonads are paired structures consisting of a large number of profusely branching tubules situated within the layer of connective tissue immediately beneath the epithelial covering of the body. In a well-developed organ the ramifying tubules concresce along the dorsal side, forming one continuous layer investing the visceral mass. Two distinct systems of genital canals and two gonoducts, one on each side of the body (Fig. 1, *GD*) remain the only signs of the paired condition of the gonad. The thickness of the gonad layer, measured in transverse section through the anterior part of the body at the level of the stomach, varies from year to year according to the number of cells produced during the winter and spring. In cases of extraordinarily prolific development this creamy and well-defined zone may reach over 20 mm. in thickness in an adult oyster. It may be less than 1 mm. in a poorly developed gonad. It is not clear what conditions are responsible for these differences, which may occur from year to year in the same locality and in the same stock of oysters. This problem calls for special investigation which is beyond the scope of the present work.

In a well-developed gonad of an oyster, the branching and twisted tubules are crowded with sex cells. A few follicles can be found, the lining of which still consists of a single layer of undifferentiated germinal epithelium or of cells at various stages of spermato- and ovogenesis. The wall of each follicle is made of a thin layer of connective tissue fibres. The outermost portion of the gonad, adjacent to the surface epithelium, is occupied by an anastomosing system of genital canals which in a ripe specimen are easily recognizable with the naked eye. They form an arborescent structure (Fig. 1, *GC*) in which the branches increase in diameter toward the posterior end of the gonad.

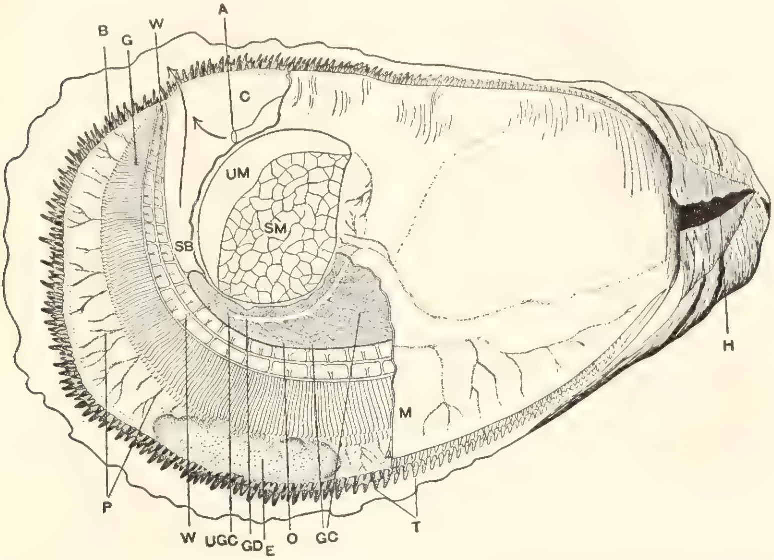

FIG. 1. Female *Ostrea virginica*, right shell and part of the mantle fold removed. The wall of the suprabranchial chamber dissected and gills pulled down to show the ovary and the water tubes of the gill lamellæ. × 1.

A, anus; *B*, border of mantle; *C*, cloaca; *E*, eggs passed through the gills and accumulated in the pallial cavity; *G*, gills; *GC*, genital canals; *GD*, gonoduct (oviduct); *H*, hinge; *M*, mantle; *O*, ovary; *P*, pallial cavity; *SB*, suprabranchial chamber; *SM*, adductor muscle, portion with striated fibres; *T*, tentacles; *UM*, adductor muscle, portion with smooth fibres; *W*, water tubes of the gills; *UGC*, urinogenital cleft. Arrows indicate the direction of the cloacal current.

The genital canals persist throughout the year and constitute the primary gonads, which, by the growth of their inner walls and a series of evaginations, give rise to the tubular secondary follicles. In transection the lumen of the genital canal appears as a narrow space between a thin body wall, consisting of a layer of connective tissue covered with epithelium, and a dense mass of ovarian or testicular follicles. The portion of the lumen adjacent to the body wall is lined with a ciliated epithelium while its opposite side, i.e., the inner arch of the lumen, is lined with germinal epithelium. Scattered muscle fibres are found here and there in the connective tissue of the gonad but are slightly more abundant in the body wall exterior to the genital canals. In both sexes the canals converge on each side of the gonad into a very short gonoduct (oviduct or spermiduct) through which the sperm or ova are discharged into a urinogenital cleft (*UGC*). In an adult oyster about 4 inches long, examined by the author, the oviducts did not exceed 0.15 mm. in length while the spermiducts were about 0.75 mm. long. When the

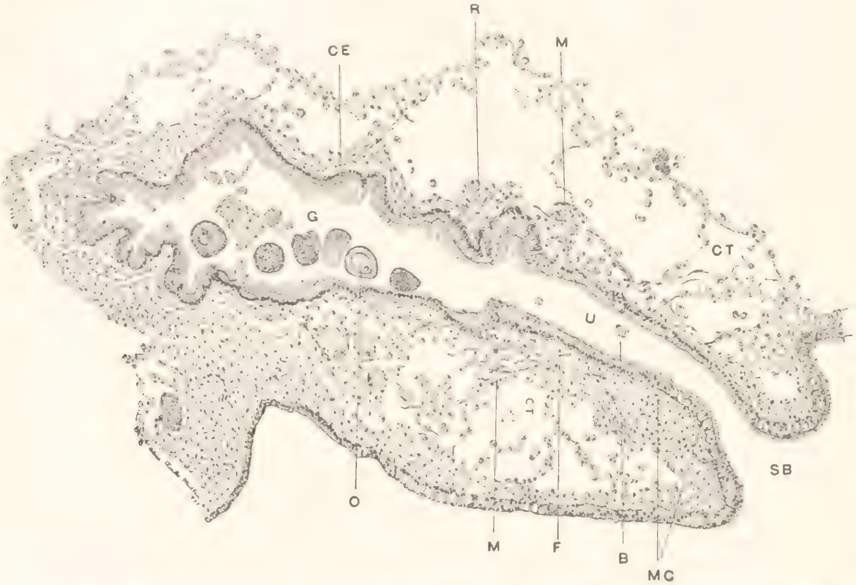

FIG. 2. *Ostrea edulis*, transverse section of the right urinogenital cleft and oviduct preserved during ovulation. Camera lucida drawing × 150. Hæmatein-eosin.

B, blood cells; *CE*, ciliated epithelium; *CT*, connective tissue; *F*, connective tissue fibres; *G*, oviduct; *M*, muscle fibres; *MC*, mucous cells; *R*, ridge of ciliated cells separating the oviduct from the urinogenital cleft; *SB*, suprabranchial chamber; *O*, ripe eggs discharged from ovary; *U*, urinogenital cleft.

gonoduct is empty its lumen has a slit-like appearance with the ciliated lining folded in numerous ruffles (Fig. 2). In the ducts filled with eggs or sperm, the walls are greatly distended and the epithelial lining is stretched (Fig. 3).

The lumen of the gonoducts is completely lined with ciliated columnar epithelium which rests on a basal membrane surrounded by a layer of connective tissue fibres. The absence of germinal epithelium distinguishes the gonoducts from the genital canals although the transition from the canal to the duct is gradual. Circular and oblique muscles are scattered in the walls but do not form a continuous sheathing. In preparations stained with hematoxylin and eosin, the connective tissue fibres can easily be mistaken for muscle fibres but their true nature is clearly revealed in sections treated with the differential triple stain of Mallory which stains the connective tissue blue. Roughley (1933) states that in *Ostrea commercialis* the gonoducts are surrounded by bands of sphincter-muscle fibres immediately internal to the cleft. No such structures have been found in *O. virginica* although, as stated above, a few scattered muscles occur in the walls of the gonoducts.

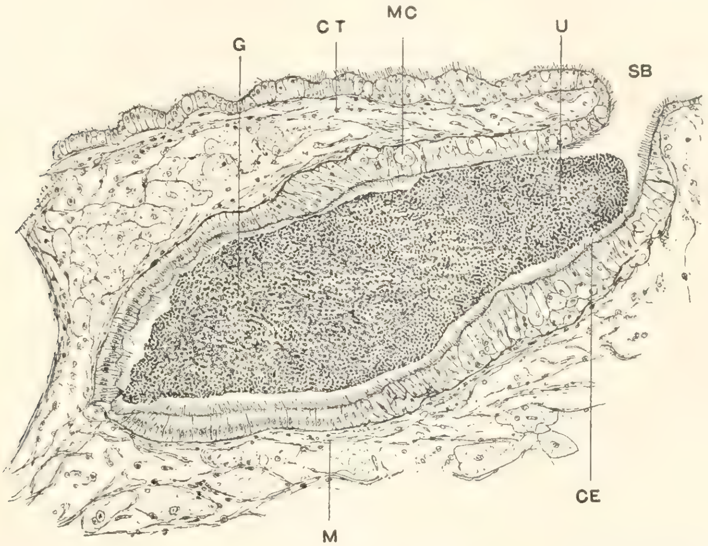

FIG. 3. *Ostrea edulis,* transverse section of left urinogenital cleft and spermiduct preserved during ejaculation. Both spermiduct (*G*) and the urinogenital cleft (*U*) are filled with ripe sperm. Camera lucida drawing × 150. Hæmatein-eosin.

CE, ciliated epithelium; *CT,* connective tissue; *G,* spermiduct; *MC,* mucous cells; *M,* muscle fibres; *SB,* suprabranchial chamber; *U,* urinogenital cleft.

Unfortunately no illustration of the sphincter is given by Roughley and a detailed comparison between the gonads of the two species of oysters is therefore impossible.

The urino-genital cleft into which the gonoducts open anteriorly and independently of the ureter is a narrow slit on the surface of the gonad (Fig. 1, *UGC*). A delicate membrane of connective tissue covered on both sides with ciliated epithelium forms the external portion of its wall. Histologically the cleft differs from the gonoduct only in the character of its epithelial lining which contains large numbers of mucous cells absent in the gonoduct (Figs. 2, 3, *MC, CE*). In the female the opening of the gonoduct into the cleft is marked with a well-pronounced ridge of tall ciliated cells not noticeable in the male (Fig. 2, *R*). On each side of the oyster the urinogenital cleft opens into a suprabranchial chamber in which a strong outgoing stream of water is maintained by the ciliary motion of the gill epithelium.

From an anatomical and histological study of the gonad it may be inferred that eggs and sperm are discharged primarily by the motion of the cilia of the epithelial cells lining the genital canals and gonoducts and that the contraction of the muscle fibres scattered between the follicles and in the walls of the canals is of lesser importance and probably only facilitates their release from the follicles. Judging by the distribution of the muscle and connective tissue fibres in the walls of the gonoducts, it appears possible that their contraction may to a certain extent constrict the lumen and thus impede the emission of sperm or ova. The muscle fibres appear to be better developed in the walls of the spermiducts than in the oviducts (Figs. 2, 3, *M*). Their possible rôle in ejaculation is discussed later.

Spawning Reactions of the Female

The spawning of the female may be divided into three distinct phases: the discharge of eggs from the ovary (ovulation), the rhythmical contractions of the adductor, and the closing and opening of the mantle. Of these the first phase is obviously the principal sexual reaction of the organism, while phases two and three may be considered as accessory reactions providing a mechanism for the dispersal of eggs. Spawning may be observed in a ripe female without employing special apparatus or procedures. For observation the oyster is placed in a small glass dish filled with warm sea water (25–27° C.). When the shell opens and the mollusk begins to feed, one can easily notice that the opposite borders of the mantle are kept sufficiently apart to permit free access of water into the pallial chamber. Under ordinary circumstances this position of the mantle is maintained for a long time with only occasional changes (Hopkins, 1933, p. 482, Fig. 7, *A*). An approaching spawning is marked, however, by an unusual muscular activity along the edges of the mantle and by a change in the position of its inner borders and tentacles. The opposite folds which previously were flattened and kept almost parallel to the surface of the shell, now occupy positions perpendicular to it. Contractions of the mantle and the corresponding changes in the positions of its borders begin simultaneously from the posterior and anterior ends and spread toward the middle. The process continues for several minutes. During this time the borders may come in contact with one another and separate again. Finally they assume such a position that the pallial cavity is almost completely closed, only a small opening being left at a place approximately equidistant from the mouth and cloaca.

In the majority of cases the tonus level of the adductor muscle changes during this time and the valves open more widely. Then the adductor begins a series of contractions which continue until the end of spawning. Coincident with the relaxations of the adductor and gaping of the valves, a white cloud of ova appears at the anterior part of the pallial chamber (Fig. 1, *E*) and, descending posteriorly, fills it. The ova accumulate in front of the small opening left between the two opposite borders of the mantle and by sharp contraction of the adductor are expelled into the water (Fig. 6). Almost immediately the adductor relaxes, the valves open again, the borders of the mantle come together leaving the opening at approximately the same place it occupied before, and a new batch of eggs, which in the meantime has entered the pallial chamber, is expelled. The process repeats itself with great regularity and may continue for more than one hour, its duration depending on the amount of ripe ova in the gonad. Gradually the discharge of eggs ceases; the contractions of the adductor become weaker and irregular, and the reaction reaches an end.

The activity of the mantle during spawning is less important than the contraction of the adductor for in many instances, especially when only a small number of eggs are discharged, the mantle borders remain flattened and the pallial cavity is open along its entire length. It is evident that the formation of a small aperture, although helpful in producing better dispersal of eggs, is not indispensable to the success of spawning.

For a more detailed study of the behavior of the adductor muscle during spawning the following technique was used. An oyster immobolized on its left side with a mixture of plaster of Paris and cement was placed on a suitable support in a glass tank containing 27.5 liters of sea water. The right valve was attached to a lever which recorded the shell movement on a slow-motion kymograph. Temperature was maintained constant within ± 0.1° C. by means of a heater, stirrer, and mercury temperature controller, operated through a relay. The entire set was mounted on an adjustable base supported by a tripod. Water was continually aerated or, if necessary, was kept running. The lever was arranged in such a way that the closed positions of the shell corresponded to the peaks of the kymograph curve. By this method several hundred records of shell movement were obtained. Those reproduced in this paper are the photographic prints of the originals.

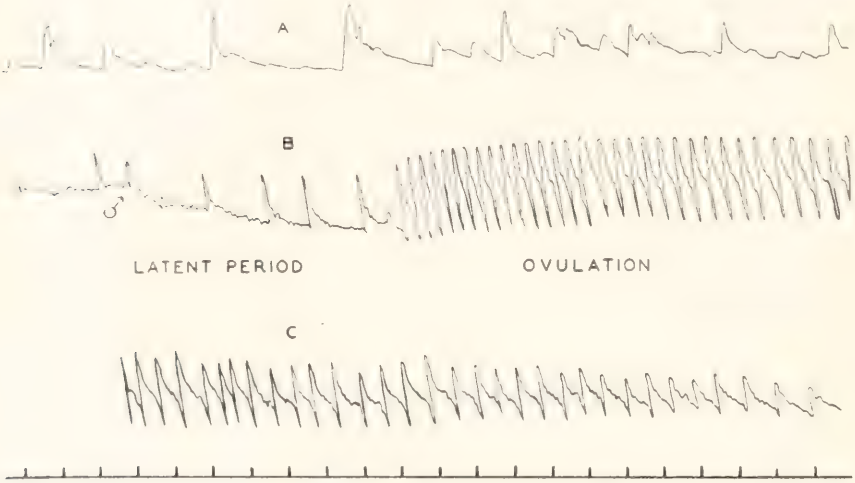

FIG. 4. *Ostrea virginica,* spawning reaction of a female. *A,* normal muscular behavior during feeding; *B* and *C,* spawning reaction. Time interval, 1 minute (bottom line). Note the change in the tonus level during the latent period. ♂ indicates time when live sperm was added to the water.

The closing of the shell of an oyster is affected by the contraction of the adductor muscle; its opening is due to the elasticity of the hinge ligament which acts as a spring forcing the valves apart when the adductor relaxes. Spontaneous shell movements are therefore entirely controlled by the contractions and relaxations of the adductor, whereas the purely mechanical action of the hinge depends exclusively on the expansion of the compressed elastic material of which it is composed. Under ordinary conditions the shell movements of an oyster are rather irregular (Fig. 4, *A*). Occasional strong contractions of the adductor resulting in a complete closure of the valve are followed by periods of relaxation interrupted by one or several light contractions causing partial closing of the shell. Strong contractions are either ejection reactions by which the oyster gets rid of the material accumulated in the branchial chamber, or are responses to a great variety of chemical and physical stimuli; as for instance—mechanical disturbances, sudden change in the intensity of illumination, chemical irritation by acids, salts, and various other organic and inorganic compounds. The magnitude of the reaction in these cases is usually directly proportional to the intensity of the stimulus.

Partial closure of the valves is caused by the contraction of the striated part of the adductor muscle, while the maintenance of the valves in a given position against the pull of the hinge is attributed to the smooth component or so-called "catch" muscle. Under normal environmental conditions shell movements are characterized by long relaxation periods which vary from two to several minutes and are often interrupted by secondary contractions. A comparison of the records obtained during feeding with those performed at the time of spawning, reveals a great difference in the muscular behavior. The approach of ovulation is very often accompanied by a change in the tonus level (Fig. 4, *B*) and greater relaxation of the adductor. Then begins a series of rapid contractions and relaxations following one after another with remarkable regularity and continuing for a considerable time. Constancy in the amplitude of the relaxation curve, especially during the first half of the reaction, and the remarkable rhythmicity of the contractions are the most distinctive features of the sexual reaction of the female. These phenomena do not occur under any other circumstances. Attempts to reproduce this type of reaction in non-spawning females by physical stimulation (pricking, electric current) or by applying various drugs and chemicals, have been unsuccessful. Rhythmical contractions of the striated component of the adductor in *O. gigas* were described by Hopkins (1936, p. 500, Fig. 1), who noticed their relations to the discharge of water from the cloaca and suggested that the impulses of the activity originate within the digestive system. That the rhythmicity of muscular behavior can be due also to other causes is obvious from the observations on oysters left for several hours without aeration or changing water (Fig. 5). In these cases a peculiar behavior of the adductor is probably caused by the accumulation of CO_2 and other products of metabolism for the normal activity is restored as soon as water is renewed. Injection of 1:10,000 solution of adrenalin into the pallial cavity also results in the rhythmical contraction which, however, lasts only for a short time (Fig. 5, *C*). All these contractions have longer relaxation periods and are less regular than those associated with the sexual activity of the oyster. This can easily be noticed by comparing the records reproduced in Figs. 4 and 5.

The question may be raised whether the behavior of the adductor during spawning represents ejection reactions in response to irritation caused by the presence of ova in the pallial chamber. That this is not the case is indicated by the continuation of typical spawning reactions after the discharge of ova has ceased. Furthermore, the quick relaxation, which always precedes a sharp contraction of the adductor during spawning, begins before the eggs appear in the pallial chamber and the amplitude of the contraction is entirely independent of the amount of the discharged material. It is typical of the spawning reactions that the contractions begin with the maximum amplitude of the up and down strokes (Fig. 4), although at the very beginning only a few ova are discharged. If the rhythmical contractions were only ejection reactions comparable to the oyster's response to irritation caused by the accumulation of foreign material in its pallial chamber, one would expect that the amplitudes of the curves would have varied with the amount of material expelled. All the records obtained during the present investigation show, however, that the strokes of the greatest amplitude occur at the beginning of the reaction and remain constant for a considerable period of time. The supposition that the contractions of the adductor during spawning are ejection reactions is therefore untenable. It may be inferred that the adductor of the ovulating female receives specific

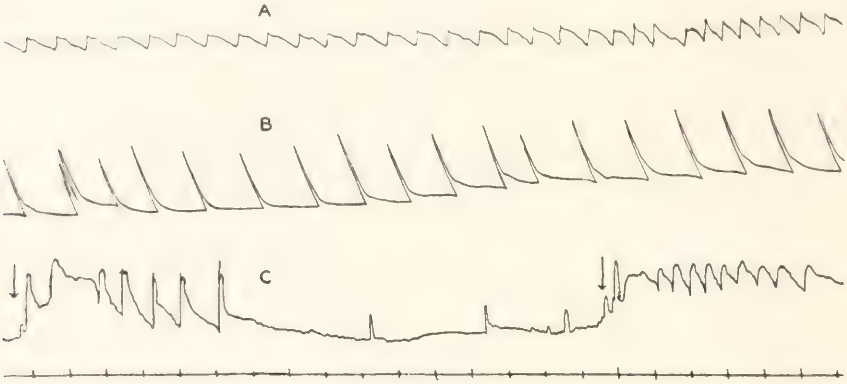

FIG. 5. Non-spawning rhythmical contractions in female oysters. *O. virginica* (*A*) and *O. gigas* (*B*) kept for several hours without aeration. *C*, effect on adductor muscle produced by 1 : 10,000 adrenalin solution injected into pallial cavity of *O. virginica*. Time of two treatments indicated by arrows. Time interval, 1 minute (bottom line).

internal stimuli which control its action, and which are present only during ovulation.

At the beginning of spawning the adductor muscle may contract as many as three times a minute but gradually the rhythm slows down, the contractions become less frequent and less regular; the duration of the relaxation periods increases, and the muscle resumes its normal activity. Sometimes the change takes place so gradually that the end of the reaction can not accurately be determined.

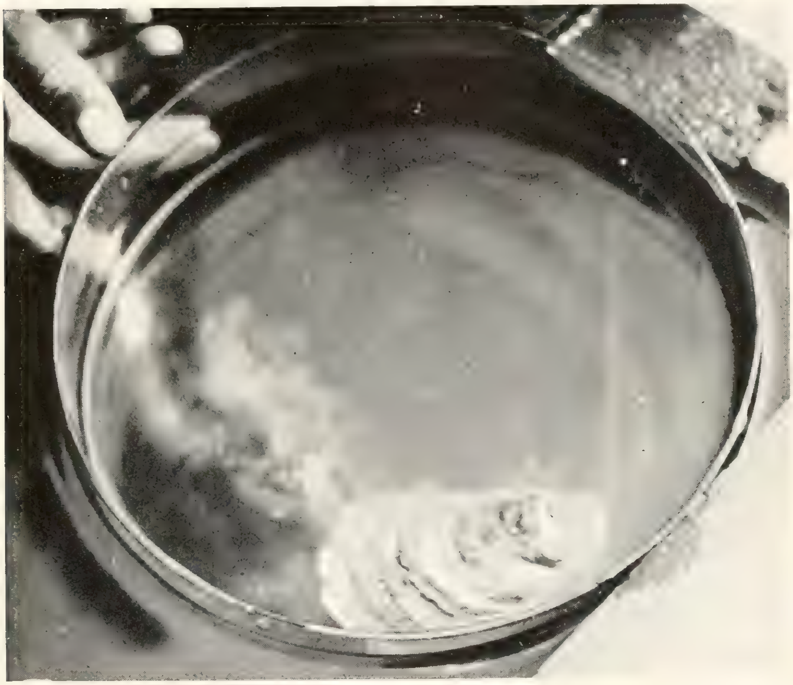

Fig. 6. Female *O. virginica* in the act of spawning. The photograph was taken at the moment the eggs were discharged by snapping of the valves. Note near the edges of the shell, a narrow jet of eggs expelled through a small opening between the borders of the mantle.

Kymograph records show that during spawning the relaxation periods are interrupted by brief pauses which give the curve a ladder-like appearance usually more pronounced toward the end of the spawning (Fig. 4, *C*). As will be shown later, this second half of each relaxation period coincides with the appearance of ova in the pallial chamber.

Contractions of the adductor, plus the action of the mantle, provide an efficient mechanism for the dispersal of ova in the surrounding water. The small hole between the two borders of the mantle through which the ova are ejected, produces the same effect as a narrow nozzle at the end of a garden hose, considerably increasing the zone of their dispersal. Spawning females taken out of the water were observed to shoot their spawn to a distance of about four feet. Since fertilization takes place outside the organism and since the ova, being heavier than water, quickly settle on the bottom, the biological significance of this arrangement and the advantages it provides for the propagation of the species are obvious.

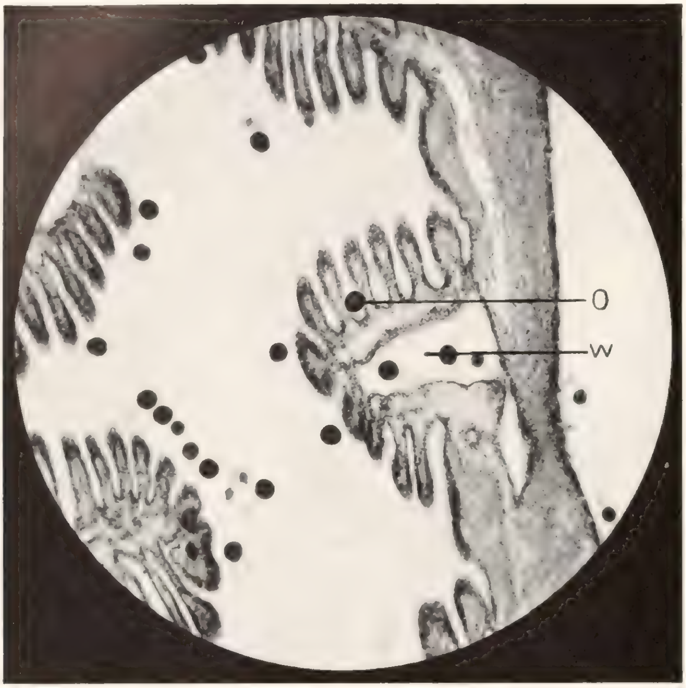

FIG. 7. Transverse section of the gills of the female *O. virginica* killed during spawning. Photomicrograph × 88. Hæmatein-eosin. Note the presence of eggs in the water tube, *W*, and in the ostium, *O*.

During ovulation ripe eggs are discharged through the oviduct and urinogenital cleft into the suprabranchial chamber and cloaca. Their passage from the ovary and through the ducts can easily be observed in a living organism. For this purpose a spawning female is taken out of the water, one of its valves is removed, and the urinogenital cleft exposed by making an incision in the wall of the cloaca and suprabranchial chamber. The oyster is immediately returned to the water. The passage of eggs along the genital canals, oviduct and through the cleft can now easily be observed with a magnifying lens. Ova expelled by the ciliary action of the epithelial lining of the ducts form a continuous stream and settle in the exposed part of the cloaca. Some of them are carried away by the cloacal current. Ovulation, once started, continues without interruption for a long time and is not stopped even by severe injury to the organism. The contractions of the severed adductor muscle persist but are not correlated with the delivery of eggs from the ovary. There are no noticeable contractions in the walls of the genital ducts or in the urinogenital cleft.

The question naturally arises as to how, in the intact organism, the ova discharged into the suprabranchial chamber reach the pallial chamber from which they are completely separated by the gills and walls of the branchial chambers (Fig. 1). Investigations of Kellogg (1892); Yonge (1926); Galtsoff (1928); Hopkins (1933) and others show that water is pumped from the pallial chamber through the ostia and water tubes of the gills into the suprabranchial chamber from whence it is expelled into the cloaca. In *O. virginica* a portion of it goes into an asymmetrical promyal chamber. In an actively feeding oyster a stream of water can easily be noticed at the dorso-posterior and dorso-anterior sides of the body. One would naturally expect that every particle introduced into the suprabranchial chamber would be carried away with one of these streams (Fig. 1). Yet the ova of a spawning female find their way into the pallial chamber against the current produced by the gills and are expelled from the posterior side of the body (Fig. 6).

Observations on ovulation in females in which the valve has been removed and the cloacal wall has been dissected, show that the only way the eggs discharged into the suprabranchial chamber can reach the pallial cavity is through the water tubes and ostia of the gills. This conclusion is fully corroborated by examination of sections of female oysters killed during the act of spawning in which unfertilized ova were found inside the water tubes and near the ostia (Fig. 7, *W*, *O*). Since the beating of the lateral cilia throws the suspended particles toward the outer surface of the gill lamellæ (Galtsoff, 1928) where they are caught by the frontal cilia and eventually conveyed toward the mouth, the presence of an egg just underneath the lateral cilia is good evidence that it has reached this place from inside through the water tubes and suprabranchial chamber. This conclusion is fully supported by experiments in which the passage of eggs through the gills was observed in vivo. The following technique has been developed for making such observations. A piece of shell of a ripe female was sawed off and carefully removed avoiding any injury to the tissues. After the operation, the underlying piece of mantle usually rolled up exposing the gills (Fig. 8, *A*). The oyster was then placed in a finger-bowl filled with sea water

(25° C.) and ovulation was stimulated by the addition of sperm (Galtsoff, 1930*b*). The shell movement was recorded on a kymograph by means of a lever attached to the remaining portion of the valve. The exposed gills were illuminated by a strong light and examined under a binocular microscope mounted on a suitable stand. Under these conditions the passage of eggs through the gills could easily be observed.

It has been noticed that they emerge from the ostia in the area which extends from the anterior part of the gills for about two-thirds of their length. The greater number of eggs pass along the proximal half of the gill lamellæ, although some of them escape through the distal portion of the gill. This experiment has been repeated several times and each time the moment of the appearance of eggs on the surface of the gills was marked on the kymograph curve. The emergence of eggs through the ostia coincided with the maximal gaping of the valves and was therefore associated with the rhythmical contractions of the adductor.

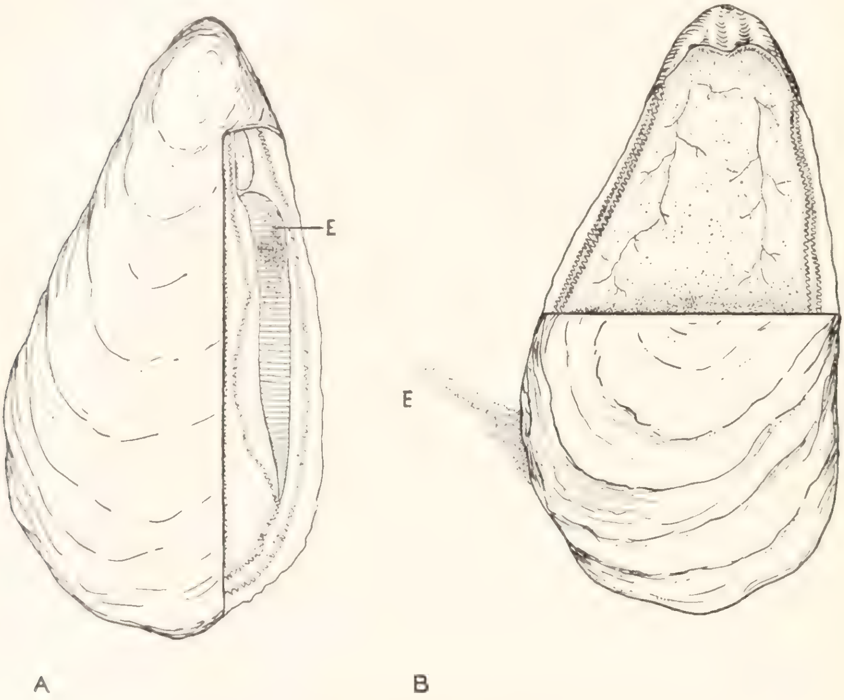

FIG. 8. *Ostrea virginica*, passage of eggs through the gills. *A*, portion of shell is sawed off and the mantle rolled up. Eggs (*E*) pass through the anterior part of the gills. *B*, anterior half of the shell is removed but the adductor is intact. Eggs are discharged through the cloaca.

What forces the eggs to take the course through the narrow passages of the water tubes against the current produced by the gill epithelium? Several possible factors have been considered: a reversal of ciliary motion in the gills, suction caused by the rhythmical movements of the valves, and difference in pressure between the suprabranchial and pallial chambers. While the evidence is not sufficiently conclusive to permit a definite answer to the question, certain of these possibilities may be ruled out.

The possibility of the reversal of ciliary motion cannot be verified by observation because the action of the lateral cilia cannot be observed in the intact gill lamella. The beating of the frontal cilia may, however, be observed by noting the movement of particles of carmine powder. During the passage of eggs it appears to be normal. If there is a reversal of ciliary motion it is therefore limited to the lateral cilia. The possibility of a reversal of the lateral cilia may not be completely excluded, but confirmatory evidence is lacking. Nelson (1936) states that the rate of pumping of water greatly decreases during the spawning of the female oyster. This may be indicative of a disturbance in the functioning of the lateral cilia. Another explanation is possible, however, for we know that the mantles of the spawning female reduce the opening, or even almost close the pallial aperture. This action obviously will decrease or completely stop the exchange of water. The reduced rate of pumping during spawning does not, therefore, mean that the beating of the lateral cilia ceased or was reversed. Other observations cast further doubt on the reversal of ciliary motion in the gills. Experiments were performed in which the anterior portion of the shell was cut away, preventing shell movement but leaving the muscle with adjacent part of the shell intact (Fig. 8, *B*). Because the hinge was severed, the oyster was unable to open its valves, but the adductor muscle and its nerve ganglia were in no way affected by the operation. Under these conditions eggs discharged from the ovary do not pass through the gills but are washed away from the cloaca by the respiratory current. If there were a reversal of the ciliary beating during ovulation a continuous oozing of eggs through the gills would be expected. Additional negative evidence is supplied by the fact that in several instances spawning oysters have been observed to discharge eggs both through the gills and through the cloaca.

We have already seen that the emergence of eggs through the ostia coincides with the period of maximal gaping of the valves. These facts led the author to consider whether rhythmical shell movement might cause a suction which would draw the eggs from the suprabranchial chamber into the water tubes and pallial cavity (see reference to this in Hopkins' paper, 1937, p. 457). Observations on oysters in which the gills were exposed by cutting a piece of shell, thus forcing the mantle to roll up and leave the pallial chamber wide open, make this explanation untenable.

Observations made during the summer of 1937 with sex-inverted oysters (Galtsoff, 1937) are of particular interest because they show that something more than rhythmical shell movement is necessary for the passage of eggs through the gills. In these experiments one adult female, which during the preceding summer was a male, discharged the eggs through the cloaca in male fashion in spite of the well-developed contractions of the adductor which are typical for a spawning female. Obviously some other conditions necessary to drive the eggs through the gills were still wanting.

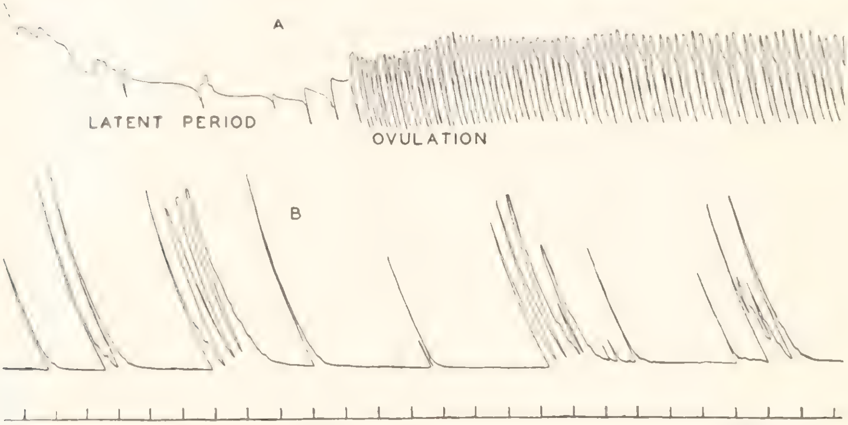

FIG. 9. *Ostrea gigas,* spawning reaction of two females. *A,* beginning of the reaction; note change in tonus level during the latent period and rhythmical contractions during ovulation. *B,* spawning reaction about one hour after its beginning; during the brief periods of contractions eggs are discharged through the gills; during the relaxation periods (horizontal parts of the curve) they are discharged through the cloaca. Time interval, 1 minute (bottom line).

The fact that the eggs are forced through the gills from the inside indicates that pressure in the suprabranchial chamber is higher than on the other side of the gill lamellæ. The increase in pressure can easily be produced by the discharge of a large amount of material from the gonad and closure of the cloacal opening. Although direct evidence is lacking, this supposition is in accord with other observed facts. Since it is known that the discharge of eggs from the ovary once started continues without interruption for a long time, it is logical to expect that pressure inside the suprabranchial chamber increases when the contraction of the adductor closes the valves and compresses the gill lamellæ. During the following relaxation and gaping of the valves the gill lamellæ spread apart and eggs which had accumulated inside are forced through the water tubes and ostia. This may be possible only if the cloacal opening remains closed for otherwise they would be carried away with the outgoing current of water. Whether the soft and contractile edges of the cloaca come together, closing the opening during ovulation, has not been ascertained by observation but the spreading and contraction of the gill lamellæ has often been noticed.

The rather complex and circuitous method of discharging eggs described for *O. virginica* occurs also in other bivalves. It is known that in many incubatory forms the ova are retained in the interlamellar branchial spaces. In *Ostrea edulis, O. angasi, O. lurida,* and *Entovalva* sp. (Pelseneer, 1906, p. 244) eggs develop in the pallial chamber outside the gills, but in the *Unionidæ* they are retained in the interlamellar branchial spaces in which special pouches or marsupia are developed (Ortmann, 1911; Lefevre and Curtis, 1910). Ovulation in the latter forms was observed in but a few cases (Latter, 1891; Ortmann, 1911, p. 298; Howard, 1914, p. 35). Latter, describing the process in *Anodonata* and *Unio,* states that "In order that the ova may reach their final resting place there must be some reversal of the respiratory currents." He could not, however, detect any reversal of the ciliary motion and attributed the passage of eggs from the cloaca into the gills to a suction caused by the gaping of the valves. This conclusion, however, was not verified by observation.

Stafford (1915) states that in *O. lurida* eggs liberated from gonoducts into the suprabranchial chambers, being too heavy to be carried in the respiratory current, flow naturally into the water tubes and are forced through them into the pallial chamber by the pressure of their mass. This explanation, apparently not based on observation, requires further verification for it is very doubtful that the ova of this species are much heavier than those of *O. virginica.* In the latter species, as has been shown above, they are easily carried away by the current. Furthermore, it seems improbable that the weight of eggs will be sufficient to counteract the current maintained in the water tubes by the ciliated epithelium.

Spawning in all three groups of bivalves mentioned above may be considered as representing three consecutive stages of one process. In *Unionidæ,* the ova penetrate into the water tubes which develop into special broodchambers, to remain there until the emergence of the glochidia; in the incubatory species of oysters, *O. edulis* and *O. lurida,* the ova completely pass through the gill lamellæ but are retained in the pallial chamber between the demi-branchs; in *O. virginica, O. gigas* and *O. commercialis,* they are discharged from the pallial chamber immediately after being forced through the gills. Here the process has reached its final development. All three different types of spawning may be attributed to a greater or lesser efficiency of the combined activity of the adductor muscle, gills, and cloaca during ovulation. A detailed physiological study of the spawning reactions of various species of *Unionidæ* and of the incubatory oysters will therefore be interesting.

Sexual Reactions of *O. gigas* and *O. commercialis*

Sexual reactions in *O. gigas* and *O. commercialis* are similar to those described for *O. virginica.* Kymograph tracings of shell movements of spawning females of these oysters show the same typical characteristics, namely, change in tonus level during the latent period, rhythmicity in the contractions of the adductor muscle and gradual fading of the reaction (Figs. 9, 10). The reaction in *O. gigas* differs slightly, however, from that of the other two species. As a rule, the Japanese oyster discharges eggs both through the gills and cloaca. In one of the experiments, for instance, muscular contractions and discharge through the gills continued for 13 minutes while ovulation and emission of eggs through the cloaca lasted 21 minutes longer. It is impossible to state which of the two methods of discharge is the principal one in this species. Eggs discharged with the respiratory current through the cloaca often come in large lumps, several millimeters long, which immediately settle on the bottom. Large numbers of eggs discharged in this manner are smothered and perish while those discharged through the gills are more widely distributed throughout the water and have a better chance to survive. No discharge of eggs through the cloaca was observed in *O. commercialis.*

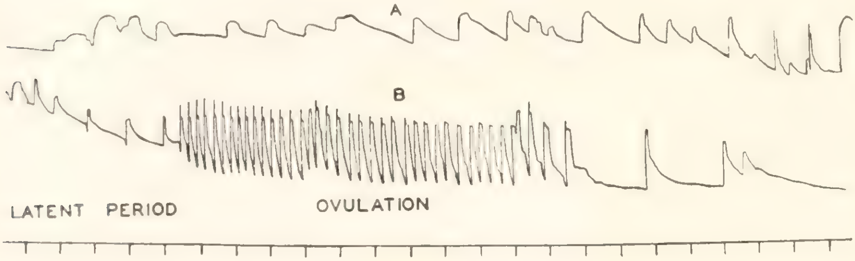

Fig. 10. *Ostrea commercialis,* spawning reaction of a female. *A,* muscular behavior during feeding. *B,* spawning reaction. Time interval, 1 minute (bottom line).

Duration and Number of Spawnings

Spawning once started may continue for a long time and cannot be stopped by taking the oyster out of the water or even by inflicting severe injury to its body. Duration of ovulation, i.e., of the discharge of ova from the gonad, cannot be measured with accuracy since the release of small numbers of eggs can easily be overlooked. Duration of the typical spawning reaction of the adductor, on the other hand,

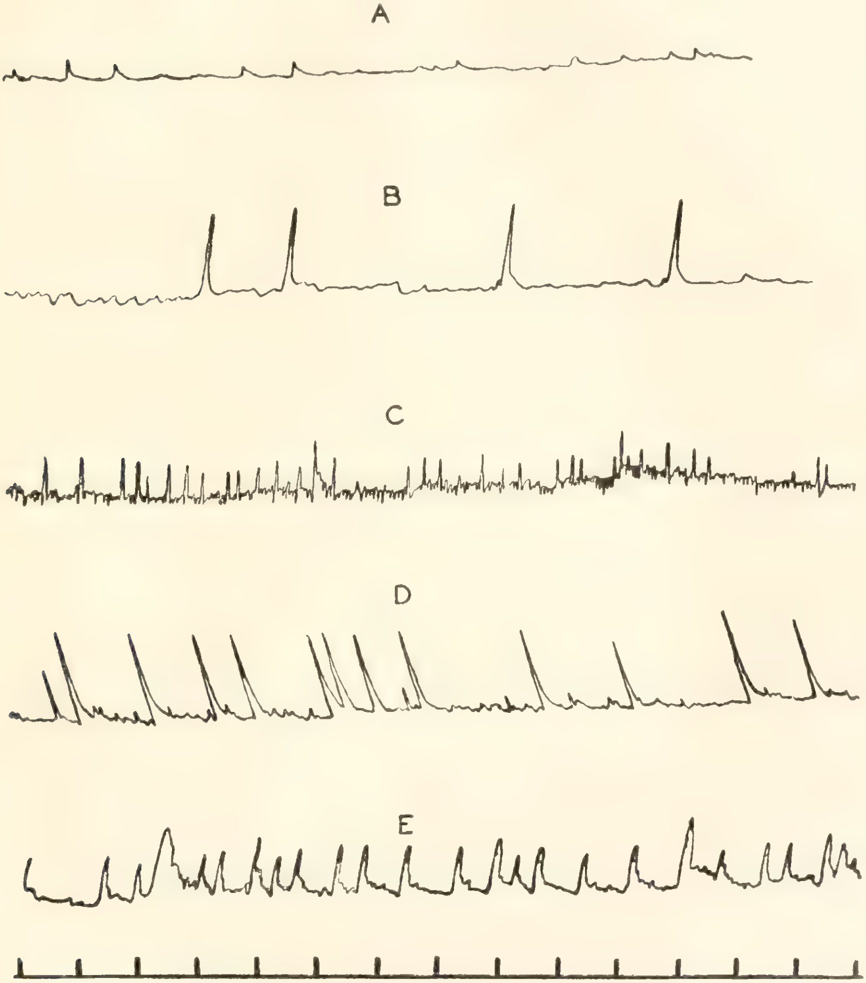

Fig. 11. *Ostrea virginica,* muscular behavior of five males (*A–E*) during ejaculation. Time interval, 1 minute (bottom line).

can be determined from the kymograph records, except in the cases where the fading of the curves is so gradual that the cessation of the reaction is indistinct. In many instances, however, the curves of muscular activity show changes sufficiently sharp to indicate the end of spawning. In 101 records of *O. virginica* available for analysis, the duration of spawning varied from 4 to 118 minutes. The shortest periods were recorded either at the beginning or toward the end of the reproductive season. In *O. gigas* (13 records), the duration of the discharge of eggs either through the gills or through the cloaca varied from 15 to 130 minutes. In the two records obtained with *O. commercialis* spawning lasted 12 and 29 minutes.

Copious ovulation was observed always to be accompanied by a large amplitude of the up and down excursions of the adductor and the greater frequency of its contractions. The greatest number of eggs discharged during one spawning was found to be 114.8 millions in *O. virginica* and 55.8 millions in *O. gigas* (Galtsoff, 1930*a*). In both cases histological examination of the spawned females revealed that only a small portion of the sex cells had been released. Judging by the thickness of the gonad layer, the fertility of *O. gigas* appears to be much greater than that of *O. virginica*. There is no doubt that the number of eggs discharged during one spawning, given above, does not represent the maximal figure.

That *O. virginica* may spawn several times during one season was ascertained by experiments conducted during several summers between June 15 and August 7, at Woods Hole. Under laboratory conditions a single female was induced to spawn seven times during this period. How often an individual oyster spawns in nature has not been determined but it may be assumed that under favorable conditions it behaves in its natural environment in the same manner as in the laboratory. Repeated spawning was also observed in *O. gigas*. In the laboratory the females of this species were induced to spawn two or three times during one month.

EJACULATION

During ejaculation sperm discharged from the testis passes through the spermiduct and urinogenital cleft into the suprabranchial chamber from whence it is carried by the current of water into the cloaca and washed away. Muscular contractions of the adductor play no rôle in the release of sperm which is effected primarily by the ciliary motion of the epithelial lining of the gonad and respiratory current produced by the gills. The behavior of the adductor during ejaculation shows a great variety of conditions from almost a complete quiescence (Fig.

11, *A*) to greatly increased activity characterized by small and irregular contractions (Fig. 11, *C* and *E*). These contractions are not, however, concerned with the release of sperm.

The sudden appearance of a jet of sperm at the beginning of ejaculation gives an impression of increased velocity of the cloacal current. The question whether there is an increase in ciliary activity during ejaculation was carefully studied in a number of experiments. Two methods were employed for measuring the strength of the cloacal current. In the first series of experiments an electric drop-counting technique, fully described in a previous publication (Galtsoff et al, 1935, p. 172) was used. The method consists in recording on a kymograph each drop of water pumped by the gills through a rubber tubing inserted into the cloaca. The method completely eliminates the effects of the adductor muscle and of the mantle which may constrict the pallial aperture, making it possible to obtain measurements of the efficiency of the ciliary epithelium alone. The results of the two experiments in which continuous records were obtained before, during and after ejaculation

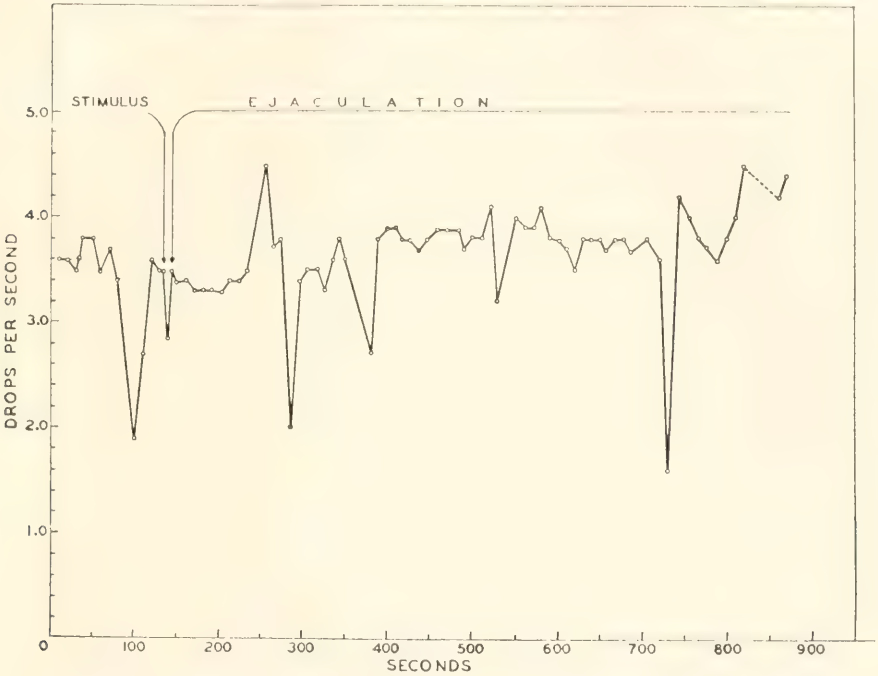

FIG. 12. Rate of pumping of water of the male *O. virginica* during continuous ejaculation. Electric drop-counting method. Each point represents 10-second average of the number of drops of water discharged by the gills.

are presented in Figs. 12 and 13. The experiments lasted 14 and 32 minutes respectively. Each point plotted on the curves represents an average number of drops per second for the preceding 10-second interval. The exact time of stimulation and of the release of sperm is indicated by the arrows. In the first experiment (Fig. 12) ejaculation lasted continuously for several minutes. There was no increase in the ciliary activity at the beginning of ejaculation and, with the exception of one high peak and four depressions, the rate of pumping remained more or less constant. Stimulation, which in this case consisted in adding thyroidin to the pallial cavity of the oyster, was followed by a temporary inhibition of the rate of pumping. This was probably due to mechanical stimulation, as a similar effect was noticeable in other cases when sea water of the same temperature was injected between the valves (Fig. 13). In the second experiment ejaculation was induced three times by the addition of oyster eggs. Not only was there no increase in the rate of pumping of water, but in two cases (Fig. 13, upper line), the rate materially decreased during ejaculation.

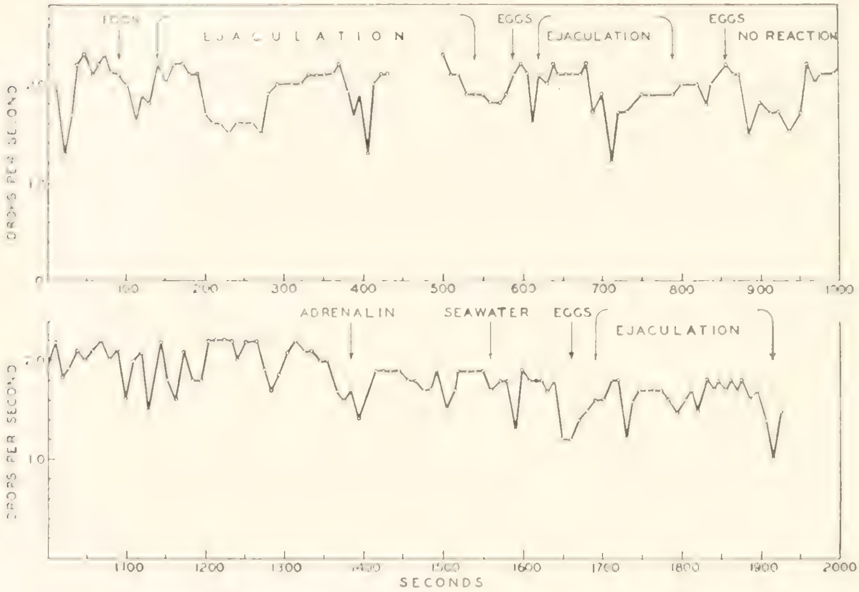

FIG. 13. Rate of pumping of water by the male *O. virginica* during and between ejaculations. Electric drop-counting method. Each point represents 10-second average of number of drops of water discharged by the gills.

Similar results were obtained by using the technique of Hopkins (1933) for recording the relative strength of the cloacal current of an intact oyster. In this method the velocity of the current is measured by the deflection of a light lever, one arm of which supports a small paper or celluloid cone placed in front of the cloacal opening. The deflections of the lever are proportional to the velocity of the current striking the cone. The method permits the study of the operations of the entire complex mechanism which controls the pumping of water by the gills. A photographic reproduction of a portion of one of the records is given in Fig. 14. The exact time of stimulation and the beginning and end of ejaculations are indicated by vertical marks (third line). The upper line represents the shell movement of the spawning male and the changes in the strength of current are shown on the second line. The uppermost points of both curves correspond to the closure

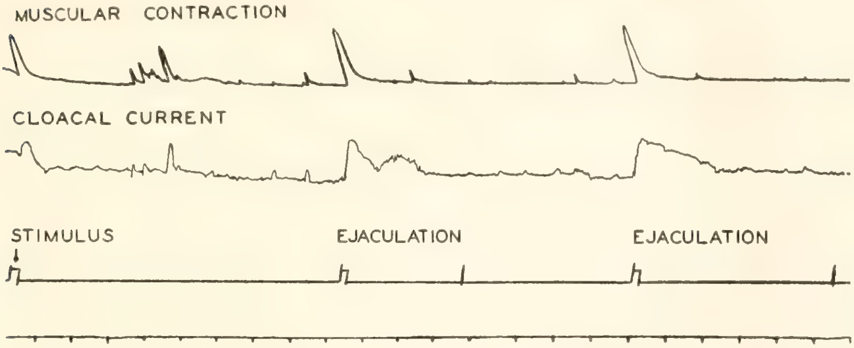

Fig. 14. Muscular contraction and relative strength of cloacal current during ejaculation of *O. virginica*. Double vertical bars (third line from top) mark the beginning of ejaculation. Single bars indicate its cessation. Time interval, 1 second (bottom line).

of shells and cessation of current. The record clearly shows a correlation between the strength of the current and shell movement and lack of significant change in the activity of the ciliated epithelium during ejaculation. The experiments repeated several times under various conditions always gave the same results indicating that sudden discharge of sperm during ejaculation is not due to the outburst of increased ciliary activity. Although no distinct sphincter is present in the spermiducts or in the urinogenital cleft, the ability of the male repeatedly to release small amounts of sperm under the influence of a proper stimulus suggests a sphincter-like action. Histological examination provides certain basis for this assumption for it shows (Fig. 3, *M*) the existence in the walls of the spermiducts of muscle fibres which are stronger and more abundant than in the oviducts. It is therefore probable that while the ciliated lining of the genital canals and spermiducts provides a motive power which conveys the ripe sperm from the testis, the release of sperm is regulated by the contractions of muscle fibres scattered in the walls of the ducts.

Ejaculation may proceed either from one or simultaneously from both spermiducts. At the beginning of the spawning season only a small amount of sperm is discharged at each ejaculation, which lasts only a few seconds, but repeated emissions can be induced immediately one after another. In several experiments a single male was stimulated to shed sperm more than a hundred times during a ten-hour period, each time releasing a small amount of spermatozoa. As the season proceeds and the amount of ripe sperm in the testis increases, each ejaculation lasts longer. Finally a stage is reached when ejaculation, once started, continues for hours and cannot be stopped until the male is completely spent.

Sperm discharged with the cloacal current remains in suspension for a considerable time and is widely distributed in the water.

The males of *O. gigas* and *O. commercialis* behave in the same manner as the males of *O. virginica.*

Summary

The gonad of an oyster consists of a large number of branching tubules containing sex cells and emptying into genital canals, the lumen of which is lined on one side with the ciliated epithelium. There are two gonoducts, one on each side of the gonad, which open into suprabranchial chambers. Spawning of the female consists in the discharge of eggs from the ovary (ovulation); rhythmical contractions of the adductor which cause opening and closing of the shell valves; and in the contraction and change in the position of mantle borders which almost completely close the pallial cavity. The last two phases of spawning are accessory sexual reactions which provide mechanism for better dispersal of eggs in the water. Muscular behavior during spawning is characterized by its remarkable rhythmicity and maintenance of a constant tonus level. It cannot be reproduced by artificial stimulation. Eggs discharged into the suprabranchial chamber are not carried away by the cloacal current but penetrate through the gills into the pallial cavity and are expelled by vigorous movements of the shell valves. Their passage through the gills coincides with the periods of greatest relaxation of the adductor and widest gaping of shell. If the shell movement is prevented by severing the hinge, eggs fail to pass through the gills and are discharged by the cloacal current. The possibility of the reversal of the ciliary motion and of the suction produced by shell

movement as the forces responsible for the passage of eggs through the gills are discussed and rejected as untenable. The following tentative explanation is offered. The discharge from the ovary and the closure of the cloaca produce an increased pressure in the suprabranchial chamber which forces the eggs through the water tubes and gill ostia into the pallial cavity. The difference in pressure on both sides of the gill reaches its maximum when the valves open and the gill lamellæ spread apart. This accounts for the penetration of eggs through the gill during the periods of greatest relaxation of the adductor.

Ejaculation is not accompanied by the rhythmical contractions of the adductor and the sperm discharged through the spermiduct is carried away by the cloacal current. There is no increase in the rate of pumping of water during spawning.

The discharge of sperm is probably controlled by the contractions of numerous muscle fibres in the walls of the spermiduct. Definite sphincter-like structure is absent.

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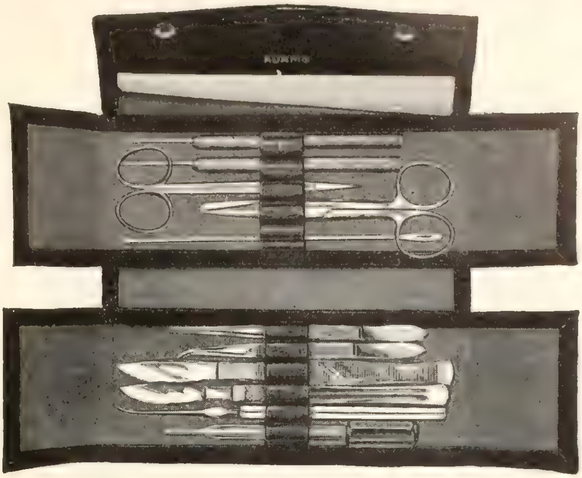

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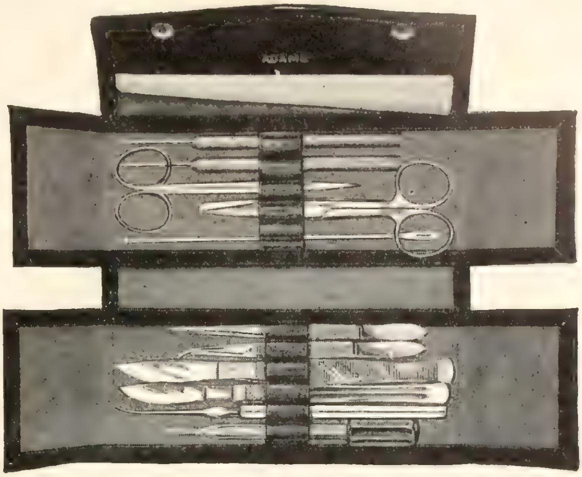

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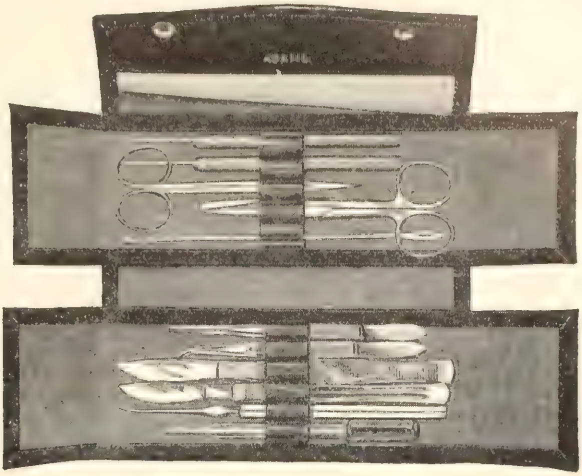

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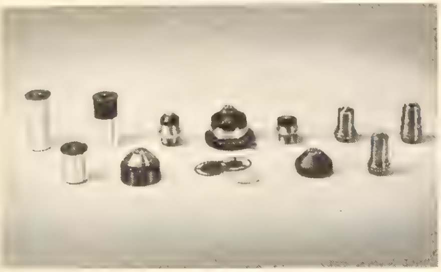

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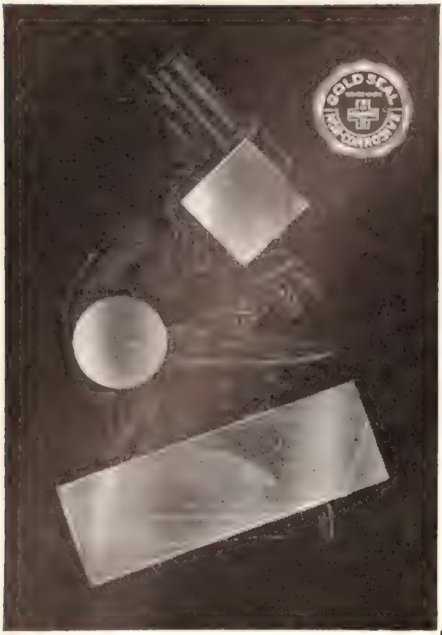

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